REMARKS

Pursuant to the requirements of 37 C.F.R. §§ 1.821-1.825, Applicants submit the enclosed Sequence Listing and computer readable form (CRF). The amino acid sequences disclosed in the specification may be found in computer readable form in file 040164.TXT on the enclosed diskette and are presented in the paper copy of the Sequence Listing, also enclosed.

Applicants hereby certify that the information recorded in computer readable form (CFR) supplied on the enclosed diskette as file 040164.TXT is identical to the written Sequence Listing. The material presented in computer readable form is not new matter because it presents sequences the same as those disclosed in the specification, as filed.

The Commissioner for Patents is hereby authorized to charge any fees which may be required to Deposit Account No. 23-0650.

The required copy of the "Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures" also is enclosed.

Applicants believe that the requirements of 37 C.F.R. §§ 1.821-1.825 have been met.

Respectfully submitted,

THE WEBB LAW FIRM

By Gwen R. Wood

Gwen R. Wood, Ph.D.
Registration No. 51,027
Attorney for Applicants
700 Koppers Building
436 Seventh Avenue
Pittsburgh, PA 15219-1818
Telephone: 412-471-8815
Facsimile: 412-471-4094
E-mail: webblaw@webblaw.com

REMARKS

The Office Action of March 31, 2005 has been reviewed and the Examiner's comments carefully considered. Claims 1-5, 15, 20-29 and 40, 41, 45-59 are currently pending in this application. Claims 6-14, and 30-38 are withdrawn from further prosecution in this application pursuant to a restriction requirement in which pending claims 1-5, 15-29 and 39-49 were elected for examination. Claims 16-19, 39 and 42-44 have been cancelled, and claims 1, 2, 4, 15, 23-26, 28, 40-41 and 48-49 have been amended, solely to promote prosecution, without prejudice or disclaimer of any previously claimed subject matter. Applicants reserve the right to file one or more continuation applications directed to the previously claimed subject matter or other subject matter disclosed in the specification. New claims 50-59 have been added. Support for the language "reduce tumor volume" contained in claim 1 can be found in paragraph 77 of the specification. Support for the language contained in new claims 51-59 can be found in claim 15 and in paragraph 24 of the specification. In view of the following Amendment, Applicants believe that all the asserted rejections are in condition for withdrawal and all of pending claims 1-5, 15, 20-29 and 39-49 are in condition for allowance.

The disclosure is objected to for failing to comply with the requirements of 37 C.F.R. § 1.821 *et seq.* regarding sequence disclosures encompassed by the definitions for nucleotide and/or amino acid sequences. Accordingly, Applicants have submitted concurrently herewith a computer readable form (C.R.F) copy of the Sequence Listing, a paper copy of the Sequence Listing and a Sequence Amendment containing the required statements as required in the "Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures."

Claims 4 and 28 stand rejected under 35 § U.S.C. 112, second paragraph, for purported indefiniteness. The Examiner asserts that the phrase "R refers to analogue substitutions" is vague and indefinite. Solely to promote prosecution, and without prejudice, this phrase has been deleted from claims 4 and 28.

Claims 1-5, 15-29 and 39-49 stand rejected under 35 U.S.C. § 103(a) for purported obviousness over Nickoloff et al. (U.S. 2002/0151487) (hereinafter "Nickoloff") in view of Shearman et al. (Biochemistry, 2000). The Examiner asserts that Nickoloff teaches a method of treating tumors or proliferative disorders and/or inhibiting angiogenesis associated with tumors, proliferative disorders or inflammatory disorders by administrating at least one

secretase inhibitor to an animal or human. Additionally, the Examiner asserts that compounds which inhibit proliferation of skin cells, as taught by Nickoloff, intrinsically read upon inhibiting angiogenesis in such skin cancer cells. The Examiner further asserts that, although Nickoloff does not teach the use of L-685,458, Shearman et al. teach that L-685,458 is a γ-secretase inhibitor.

Claim 1 as amended is directed to a method of treating a tumor in an animal or human in need of such treatment by inhibiting angiogenesis, comprising administering to the animal or human a therapeutically effective amount of a composition comprising a carrier and at least one secretase inhibitor effective to inhibit angiogenesis in said animal or human. Claim 25 as amended is directed to a method of inhibiting angiogenesis and reducing tumor volume in an animal or human in need of such inhibition, comprising administering to the animal or human an effective amount of a composition comprising a carrier and at least one secretase inhibitor.

There is no disclosure or suggestion in Nickoloff, either alone or in combination with Shearman et al., to administer a secretase inhibitor in an effective amount to inhibit angiogenesis, as recited in the claims. There is further no suggestion in Nickeloff, alone or in combination with Shearman et al., of the specific embodiments claimed in new claims 50-58, which are directed to the treatment of a human brain adenocarcinoma tumor, a human lung adenocarcinoma tumor, a human glioblastoma tumor, a human malignant breast tumor, a human malignant colon tumor, a human malignant kidney tumor, a human malignant bladder tumor, or a human malignant head or neck tumor, by administering a secretase inhibitor in an effective amount to inhibit angiogenesis.

There is no suggestion in, or motivation from, the teachings of Nickoloff that would have led one of ordinary skill in the art to administer a secretase inhibitor to inhibit angiogenesis or to directly affect tumor volume. Rather, Nickoloff teaches away from the claimed methods.

Nickoloff is directed to methods for treating skin disorders. Nickoloff states at paragraph 0010 that the invention provides "a method of inducing differentiation of an epithelial cell" and "a method for inducing formation of a barrier within epithelium", by administering a Notch agonist. Nickoloff further states that a Notch antagonist or agonist can be "provided to at least one malignant or pre-malignant epithelial cell such that the Notch pathway is activated or attenuated in the cell to retard its progression toward malignancy"

(paragraph 0010 of Nickoloff). Nickoloff provides a speculative list of a wide range of different categories of Notch agonists and antagonists that might be useful (see e.g., paragraphs 0016-0022). Example 9 of Nickoloff discloses that a gamma secretase inhibitor reduced the invasive behavior of melanoma cells in an *in vitro* assay, but this assay was limited to observing a reduction in the aggressive movement of melanoma cells through a gel filter, and there was no demonstration of inhibition of tumor growth or anti-angiogenesis. One skilled in the art and considering Nickoloff would not have been motivated to practice the claimed methods of use of secretase inhibitors to inhibit angiogenesis.

Nickoloff suggests that Notch agonists can be used for the treatment of skin cancers. From this disclosure, however, there is no suggestion of the specific discovery by the Applicants that secretase inhibitors can be used to inhibit angiogenesis. Activity of a compound as an anti-cancer agent does not necessarily mean that it is anti-angiogenic. Some agents shown to exhibit a beneficial effect as anti-tumoral agents in model systems are in fact pro-angiogenic. (See Secchiero et al., Neoplasia, 2004, 6(4):364-73, attached as Exhibit A). As described in Secchiero et al., tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo-2L is a potential anti-cancer therapeutic agent for its ability to induce apoptosis in a variety of cancer cells, but also may play a role in promoting angiogenesis.

The Examiner's attention is also drawn to Leong et al., Molecular and Cellular Biology, 2002, p. 2830-2841 (attached as Exhibit B) which states that activation of human Notch 4, which is expressed primarily on endothelial cells, *inhibited* angiogenesis. Nickoloff states in paragraphs 0017 and 0021 that a γ-secretase inhibitor can be used as a Notch antagonist. Furthermore, Zimrin et al. (WO 97/45143; p. 42, lines 3-9; attached herewith as Exhibit C) found that activating Notch signaling in microvessels inhibited cell migration (an essential component of angiogenesis) whereas the opposite was true in endothelia from large vessels. As microvessels are the vessels involved with neoangiogenesis associated with tumor growth and metastasis, a skilled person in the field of oncology would have predicted and expected that inhibition of Notch activity with a gamma secretase inhibitor would *increase* angiogenesis and thus *enhance* tumor growth. The present invention inheres in the exact opposite unexpected and surprising finding, namely, that gamma secretase inhibitors (which would be expected to specifically inhibit Notch activity) substantially *decrease* angiogenesis.

Therefore, γ-secretase inhibition of Notch signaling does not "intrinsically read upon inhibiting angiogenesis." Moreover, and more importantly, it is known that one does not have to inhibit Notch activity to inhibit angiogenesis. For example, the gamma secretase inhibitor, JLK-6, has a mode of action that is completely independent of Notch activity. Thus, the mechanism by which gamma secretase inhibitors inhibit angiogenesis may not be via inhibition of Notch signaling in endothelial cells at all but due to some other, as yet unidentified, mechanism.

Thus, a skilled artisan, in view of Nickoloff, and knowledge in the art, would not expect that a Notch agonist or a γ-secretase inhibitor would inhibit angiogenesis. In fact, Applicants state in paragraph 0063 of the present application that the anti-angiogenic activity of γ-secretase inhibitors appears to be independent of Notch cleavage. The present disclosure that γ-secretase inhibitors inhibit angiogenesis is therefore an unexpected and surprising finding.

Nickoloff provides only a single example of using a γ-secretase inhibitor, namely, to inhibit migration of tumor cells *in vitro* (Example 9). Nowhere in the example is there a teaching to use a γ-secretase inhibitor to directly treat cancer. In particular, Nickoloff does *not* disclose that γ-secretase inhibitors are anti-proliferative with regard to tumor cells in culture, or that γ-secretase inhibitors reduce tumor size *in vivo*, or that γ-secretase inhibitors are cytotoxic to tumor cells in culture or *in vivo*, or that γ-secretase inhibitors interfere with differentiation of tumor cells and cell cycling, or that γ-secretase inhibitors can reduce tumor volume and thus have a direct effect on tumor size. In short, Nickoloff merely discloses that the migration of one type of tumor cell line *in vitro* is inhibited by one γ-secretase inhibitor. Therefore, the most that one skilled in the art can learn from the teaching of Nickoloff is that a γ-secretase inhibitor can interfere with cell migration and thus potentially reduce metastasis of a tumor. Thus Nickoloff's teaching differs from the teaching of the present invention with regard to tumors in that the present invention inheres in the surprising finding that *in vivo* γ-secretase inhibitors inhibit tumor growth and reduce tumor volume *in vivo* as well as impair angiogenesis *in vitro* and *in vivo*.

Moreover, treatments for preventing or retarding progression of skin cancer do not generally rely on anti-angiogenic effects, but tend to rely on the destruction of tissue, for example, using electrical current, lasers, and physical removal of the tissue. From the

disclosure in Nickoloff of methods of treatment of skin disorders, such as skin cancers, with Notch agonists and Notch antagonists alone or in combination with Shearman's disclosure, the skilled artisan would not have been led to practice the claimed methods of inhibiting angiogenesis using a secretase inhibitor.

Nickoloff alone or in combination with Shearman et al. does not teach nor suggest the unexpected and surprising finding disclosed by the Applicant that a γ-secretase inhibitor can be administered to an animal or human in an effective amount to inhibit angiogenesis, and to treat a tumor. In particular, there is no suggestion of the treatment of a human brain adenocarcinoma tumor, a human lung adenocarcinoma tumor, a human glioblastoma tumor, a human malignant breast tumor, a human malignant colon tumor, a human malignant kidney tumor, a human malignant bladder tumor, or a human malignant head or neck tumor, by administering a secretase inhibitor in an effective amount to inhibit angiogenesis, as claimed in claims 50-58.

Applicants therefore respectfully request that this rejection be withdrawn.

Conclusion

In conclusion, Nickoloff neither teaches nor suggests, either expressly or inherently, alone or in combination with Shearman et al., the unexpected and surprising finding of the present invention, which is that animals or humans afflicted with tumors can be treated successfully with γ-secretase inhibitors to reduce tumor volume, inhibit tumor growth and inhibit angiogenesis. In view of the above amendments and remarks, reconsideration of the rejections and allowance of claims 1-5, 20-29, 40-41 and 45-59 are respectfully requested.

Respectfully submitted,

THE WEBB LAW FIRM

By [signature]

Gwen R. Wood, Ph.D.
Registration No. 51,027
Attorney for Applicants
700 Koppers Building
436 Seventh Avenue
Pittsburgh, PA 15219-1818
Telephone: 412-471-8815
Facsimile: 412-471-4094
E-mail: webblaw@webblaw.com

BEST AVAILABLE COPY

Exhibit A

RESEARCH ARTICLE

Neoplasia • Vol. 6, No. 4, July/August 2004, pp. 364–373 364

www.neoplasia.com

Evidence for a Proangiogenic Activity of TNF-Related Apoptosis-Inducing Ligand[1]

Paola Secchiero, Arianna Gonelli*, Edvige Carnevale†, Federica Corallini*, Clara Rizzardi‡, Serena Zacchigna†, Mauro Melato‡ and Giorgio Zauli†*

*Human Anatomy Section, Department of Morphology and Embryology, University of Ferrara, Via Fossato di Mortara 66, Ferrara 44100, Italy; †Department of Normal Human Morphology, University of Trieste, Via Manzoni 16, Trieste 34138, Italy; ‡Unit of Pathology, University of Trieste, Via Stuparich 1, Trieste 34125, Italy

Abstract

Starting from the observation that tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)/Apo-2L protein is expressed in both malignant and inflammatory cells in some highly vascularized soft tissue sarcomas, the angiogenic potential of TRAIL was investigated in a series of *in vitro* assays. Recombinant soluble TRAIL induced endothelial cell migration and vessel tube formation to a degree comparable to vascular endothelial growth factor (VEGF), one of the best-characterized angiogenic factors. However, the proangiogenic activity of TRAIL was not mediated by endogenous expression of VEGF. Although TRAIL potentiated VEGF-induced extracellular signal–regulated kinase (ERK) phosphorylation and endothelial cell proliferation, the combination of TRAIL + VEGF did not show additive effects with respect to VEGF alone in inducing vessel tube formation. Thus, although TRAIL has gained attention as a potential anticancer therapeutic for its ability to induce apoptosis in a variety of cancer cells, our present data suggest that TRAIL might also play an unexpected role in promoting angiogenesis, which might have therapeutic implications.

Neoplasia (2004) **6**, 364–373

Keywords: TRAIL, VEGF, signal transduction, angiogenesis, endothelial cells.

Introduction

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)/Apo-2L is a member of the TNF family of cytokines, which is broadly expressed at the mRNA level in many normal tissues and tumor cell lines [1]. TRAIL is a type II membrane protein, which can be proteolytically cleaved by cysteine proteases to a soluble form [2] as previously shown also for TNF-α and CD95 (Apo-1/Fas) ligand. The unique feature of TRAIL, compared to other members of the TNF family, is its ability to induce apoptosis in a variety of malignant cells both *in vitro* and *in vivo*, displaying minimal or absent toxicity on normal cells and tissues [3,4].

TRAIL interacts with four high-affinity transmembrane receptors belonging to the apoptosis-inducing TNF-R family. TRAIL-R1 (DR4) and TRAIL-R2 (DR5) transduce apoptotic signals on binding of TRAIL, whereas TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) are homologous to DR4 and DR5 in their cysteine-rich extracellular domain, but lack intracellular death domain and apoptosis-inducing capability and have been proposed to function as decoy receptors, protecting normal cells, including endothelial cells, from apoptosis [5,6]. Although little is known about possible nonapoptotic effects induced by TRAIL, it has been shown that endothelial cells express all TRAIL-Rs [6–8], whereas TRAIL protein is expressed in the medial smooth cell layer of the aorta and pulmonary artery [9]. Whereas cleavage of Fas ligand from the cell surface requires the action of zinc-dependent metalloproteases, generation of soluble TRAIL involves the action of cysteine proteases [2]. Notably, the vessel wall is a rich source of cysteine proteases [10], which suggests that the TRAIL/TRAIL-R system likely plays a physiological role in vascular biology. In this respect, we have recently demonstrated that addition of TRAIL to human umbilical vein endothelial cells induces the rapid phosphorylation and activation of extracellular signal–regulated kinase (ERK) and Akt [7,8]. Because these intracellular pathways are known to be involved in endothelial cell survival, proliferation, and migration [11,12], in this study, we have investigated whether TRAIL induced angiogenesis by using various *in vitro* assays. Taking into consideration that several recent studies have confirmed the hypothesis that tumor growth, in general, is dependent on angiogenesis [13,14], we have also analyzed the expression of TRAIL protein in soft tissue sarcomas because these tumors are often highly vascularized [15].

Materials and Methods

Reagents and Cells

Recombinant histidine6-tagged TRAIL was produced in bacteria and purified by chromatography on Ni^{2+} affinity resin,

Address all correspondence to: Giorgio Zauli, MD, PhD, Department of Normal Human Morphology, University of Trieste, Via Manzoni 16, Trieste 34138, Italy. E-mail: zauli@units.it

[1]This research was supported by AIRC and FIRB grants.

Received 2 November 2003; Revised 9 December 2003; Accepted 11 December 2003.

DOI 10.1593/neo.03421

as described [7]. The concentration of TRAIL used in most assays (10 ng/ml) was determined in preliminary dose–response (0.1–1000 ng/ml) experiments. For neutralization experiments, TRAIL was preincubated with TRAIL-R1-Fc and/or TRAIL-R2-Fc chimeras, according to the supplier's instructions (R&D, Minneapolis, MI). Vascular endothelial growth factor (VEGF; Peprotec, London, UK), was used at the final concentration of 10 ng/ml. Polymyxin B (Calbiochem, La Jolla, CA), was used at the final concentration of 10 μg/ml. A pharmacological inhibitor of the ERK pathway (PD98059; final concentration: 10 μM) was from Calbiochem.

Primary human umbilical vein endothelial cells (HUVECs) were obtained as described previously [8] and were used between the third and sixth passages *in vitro*. Cells were grown on 0.2% gelatin-coated tissue culture plates in M199 endothelial growth medium (BioWhittaker, Walkersville, MD) supplemented with 20% fetal bovine serum (FBS), 10 μg/ml heparin, and 50 μg/ml Endothelial Cell Growth Factor (ECGF) (Sigma, St. Louis, MO).

Neoplastic Samples and Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues of human sarcomas were obtained from 10 surgically treated patients. The tumors consisted of five liposarcomas, two leiomyosarcomas, one rhabdomyosarcoma, one angiosarcoma, and one Kaposi's sarcoma. Immunohistochemical study was performed on 4 μm sections using a streptavidin–biotin complex immunoperoxidase technique with a polyclonal anti–TRAIL antibody (Ab; clone H-257; Santa Cruz Biotechnology, Santa Cruz, CA). Isotype-matched irrelevant antibodies, used as negative control, gave absence of background. For the evaluation of tumor vascularization, sections were stained with Ab anti-CD31 (clone JC/70A; BioGenex, San Ramon, CA) and angiogenesis was quantified by assessment of microvessel density using previously described techniques [16,17]. Briefly, the number of vessel was counted throughout the entire core specimen in serial sections. Consecutive per ×20 high-power fields were examined, a median count per field was calculated, and a simple high-density/low-density score was used. Microvessel density was analyzed blinded toward the result of the TRAIL staining.

Cell Migration and Cell Invasion Assays

Cell migration was analyzed using a modified Boyden chamber assay, as described previously [18], by using 24-well plates with inserts containing 8 μm pore size gelatinized polycarbonate membranes separating the two chambers of each well (Transwell; Costar, New York, NY).

Cell invasion was investigated by using the Chemicon Cell Invasion Assay kit (Chemicon International, Temecula, CA) according to the manufacturer's instruction. This assay is performed in an invasion chamber, a 24-well tissue culture plate with cell culture inserts containing an 8-μm pore size polycarbonate membrane, over which a thin layer of extracellular matrix (ECM) is dried.

For the assays, exponentially growing cells were harvested with trypsin, centrifuged, resuspended at 0.5 $\times 10^6$ cells/ml in migration buffer [M199 medium, 10 mM HEPES, pH 7.4, and 0.5% bovine serum albumin (BSA)], and placed in the upper compartment of the chambers. TRAIL or VEGF, used alone or in combination, was added in the lower chambers. After 4 hours (for the migration assay) or 48 hours (for the invasion assay) of incubation at 37°C, cells on the upper face of the membrane were scraped using a cotton swab and cells on the lower face were fixed and stained with Mayer's hematoxylin solution. The number of migrated cells on the lower face of the filters was counted in five fields under ×100 magnification. Assays were done in triplicates.

Tube Formation Assays

In vitro formation of tubular structures was studied on BioCoat Matrigel tissue culture plates (BD Biosciences, Bedford, MA). Briefly, HUVECs were plated at 3.5 $\times 10^5$ cells/well in 24-well plates precoated with a solution of Matrigel basement membrane matrix, and left untreated or exposed to TRAIL or VEGF. After 48 hours of incubation at 37°C, the cell 3D organization was examined under an inverted photomicroscope and photographed (×40). Each treatment was performed in triplicate.

In vitro angiogenesis was assessed as formation of capillary-like structures of HUVECs cocultured with matrix-producing cells that had been UV-irradiated before plating of primary HUVECs (TCS Biologicals, Buckingham, UK) [19]. Briefly, cultures were left untreated or stimulated with TRAIL or VEGF, used alone or in combination, at day 3. When indicated, PD98059 or the vehicle (0.25% DMSO), previously diluted in medium, was added to the cultures 45 minutes before exposure to TRAIL or VEGF. Medium and treatments were replaced every 2 to 3 days. At day 12, the cells were fixed and HUVECs were stained using an anti–CD31 Ab (TCS Biologicals, Buckingham, UK), according to the instructions provided with the kit. Images were captured and analyzed. In particular, to measure the formation of the capillary network, the number of connections between three or more capillary-like structures and the total length of tubes were quantified by image analysis at ×40 magnification. Four-six different fields were analyzed per well.

Western Blot Analysis

For Western blots, HUVECs were plated in 10-cm dishes and grown at subconfluence before treatments. In order to minimize activation by serum, HUVECs were subject to partial fetal calf serum (FCS) reduction (to 0.5%) and complete growth factor withdrawal for 18 hours prior to the addition of TRAIL or VEGF, used alone or in combination. Cells were harvested in lysis buffer containing 1% Triton X-100, Pefablock (1 mM), aprotinin (10 μg/ml), pepstatin (1 μg/ml), leupeptin (10μg/ml), NaF (10 mM), and Na_3VO_4 (1 mM). Protein determination was performed by Bradford assay (Bio-Rad, Richmond, CA). Equal amounts of protein (50 μg) for each sample were migrated in acrylamide gels and blotted onto nitrocellulose filters. Blotted filters were probed with antibodies for the phosphorylated ERK1/2 and p38/MAPK (all from New England Biolaboratories, Beverly, MA). After incubation with peroxidase-conjugated

anti–rabbit or anti–mouse IgG (Sigma), specific reactions were revealed with the Enhanced Chemiluminescence (ECL) Western blotting detection reagent. Membranes were stripped by incubation in Re-Blot 1 × Ab stripping solution (Chemicon International) and reprobed for the respective total protein kinase content or β-actin (New England Biolaboratories) for verifying loading evenness.

Densitometric values, expressed in arbitrary units, were estimated by the ImageQuant software (Molecular Dynamics, Piscataway, NJ). Multiple film exposures were used to verify the linearity of the samples analyzed and avoid saturation of the film.

[^{3}H]Thymidine Incorporation

HUVECs were plated onto 96-well plates at a density of 5 ×10^3 cell/well. On the next day, the medium was changed to endothelial cell basal medium containing 0.5% FBS and 0.1% BSA (starvation medium). The cells were then pretreated with PD98059 for 1 hour and incubated with TRAIL or VEGF, used alone or in combination, for 30 hours. [^{3}H]thymidine (1 μCi) was added to each well during the last 6 hours of incubation. [^{3}H]thymidine-labeled DNA was then measured using a Beckman (Fullerton, CA) model LS6000IC liquid scintillation counter.

Statistical Analysis

Data were analyzed using the two-tailed, two-sample *t*-test (statistical analysis software; Minitab, State College, PA). Values of $P < .05$ were considered significant.

Results

TRAIL Is Expressed in Highly Vascularized Soft Tissue Sarcomas

Having previously demonstrated that TRAIL activates intracellular signal transduction pathways [7,8], which have been involved in promoting angiogenesis [13,14], in the first group of experiments, we have investigated the expression of TRAIL in some cases of malignant mesenchymal tumors because these tumors are often highly vascularized [15,20]. As shown in Figure 1*A*, the cells of a low-grade gastric leiomyosarcoma stained negative for TRAIL and were poorly vascularized. However, the malignant neoplastic cells of a high-grade leiomyosarcoma showed a strong expression of TRAIL and were characterized by prominent neovascularity. To ascertain that TRAIL expression was not confined to leiomyosarcomas, we have also analyzed TRAIL expression in some cases of angiogenetic liposarcomas (Figure 1*B*). In these sarcomas, a clear-cut positivity for TRAIL was noticed in both malignant cells as well as in tumor-infiltrating lymphocytes and plasma cells (Figure 1*B*). Finally, a high expression of TRAIL was documented also in tumors characterized by tumultuous angiogenesis, such as malignant vascular sarcomas (Kaposi's sarcoma; Figure 1*C*).

TRAIL Promotes Endothelial Cell Migration and Invasion

Because angiogenesis is a tightly regulated process, which involves the coordinated migration, differentiation, and morphogenetic organization of endothelial cells into new capillary structures [18], these aspects of angiogenesis were next examined in a series of *in vitro* assays.

To test whether recombinant soluble TRAIL could affect endothelial cell motility, HUVECs were incubated in a modified Boyden chamber with recombinant soluble TRAIL (10 ng/ml). For comparison, cells were treated also with VEGF (10 ng/ml). TRAIL significantly ($P < .01$) increased HUVEC migration (Figure 2*A*). The increase in the number of migrated cells detected with TRAIL (approximately two-fold) was similar to that observed in the presence of VEGF (2.5-fold). Interestingly, however, the simultaneous addition of optimal concentrations of TRAIL and VEGF did not show additive effects on endothelial cell migration (data not shown).

To form new blood vessels, endothelial cells have to migrate and cross basement membranes. This invasive capacity of HUVECs in response to TRAIL was investigated by measuring the invasion of an ECM layer. Addition of recombinant TRAIL significantly ($P < .01$) increased HUVEC invasion through ECM (Figure 2*B*). Again, maximal stimulation, corresponding to a 1.5-fold increase in the number of migrated cells in response to TRAIL, was similar to that observed in response to VEGF. The specificity of these biological effects was confirmed by preincubation of TRAIL with TRAIL-R1-Fc (Figure 2, *A* and *B*) or TRAIL-R2-Fc (data not shown) chimeric proteins, which completely ($P < .01$) abrogated the ability of TRAIL to promote cell migration and invasion (Figure 2, *A* and *B*), without exhibiting, by themselves, any effect on endothelial cell migration or invasion (data not shown). These data demonstrate, for the first time, that TRAIL induces endothelial cell migration and invasion through the basement membrane. Importantly, the TRAIL concentration used in these assays was in the range reported to be present in the plasma of patients affected by hematological malignancies (1–10 ng/ml) [21].

TRAIL Induces Morphological Endothelial Differentiation

To examine whether TRAIL induces morphogenetic changes resembling capillary-like structure tube formation, HUVECs were plated on 3D Matrigel plates. After 48 hours, untreated endothelial cultures showed both cells with small round shape (that remained isolated) and elongated shape, forming connections but an incomplete network of tubes (Figure 3*A*). However, cultures exposed to TRAIL (10 ng/ml) exhibited a distinct phenotype by assuming a more elongated shape, forming thin cords of inteconnecting cells (Figure 3*A*). Similar effects were also observed with VEGF (10 ng/ml) (Figure 3*A*). Morphometric quantitation of the vessel-like structures in the 3D cultures revealed that Fc-TRAIL-R1 completely abrogated this response to TRAIL (Figure 3*B*). These data demonstrate that TRAIL, like VEGF, is able to mediate morphogenetic effects leading to differentiation into vascular structures, which represent an obligatory step for the sprouting of endothelial cells and tube formation.

Because tubules formed in standard Matrigel assays are short and relatively homogeneous, the ability of TRAIL to induce angiogenesis was further investigated by using the

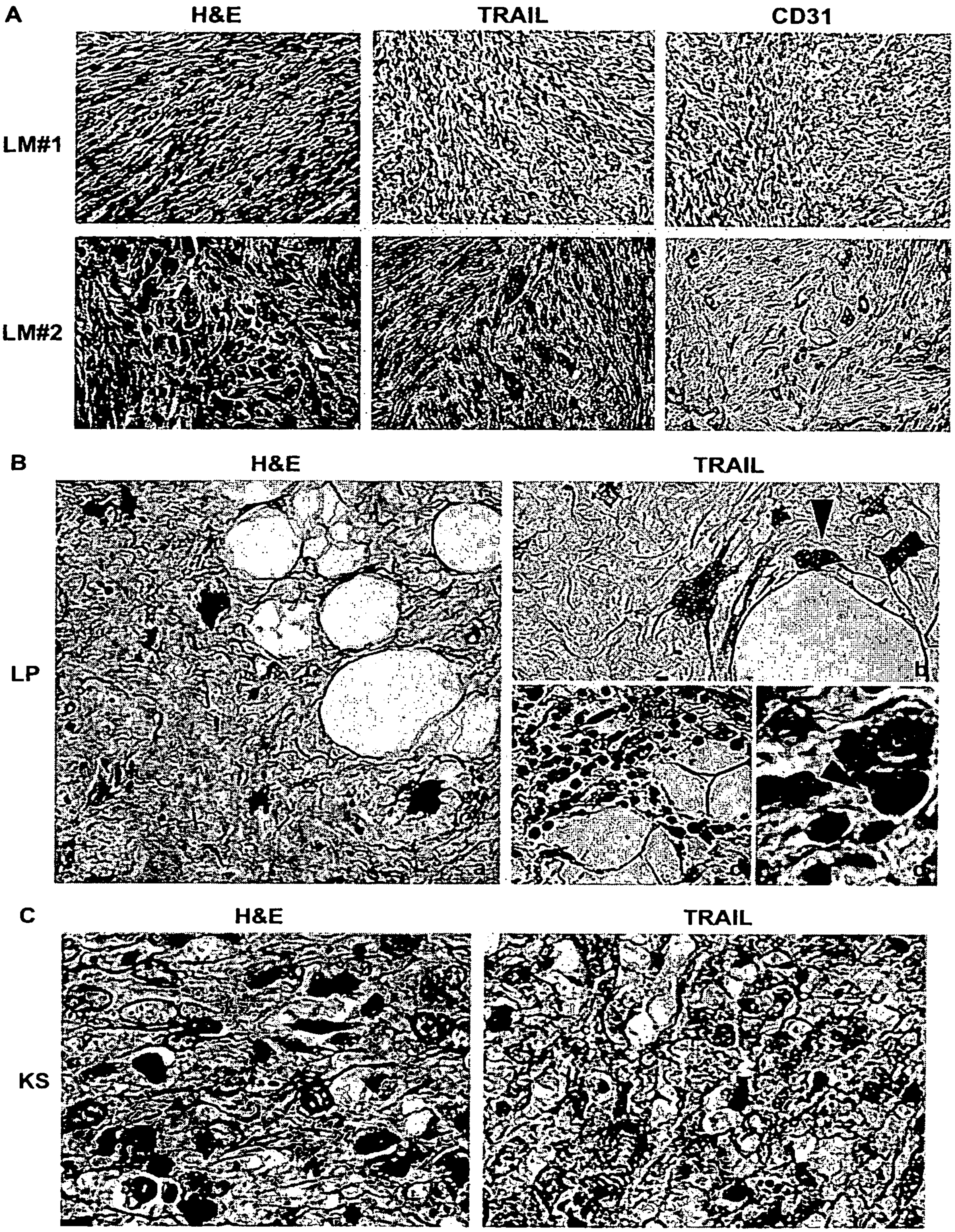


Figure 1. *Expression of TRAIL in human sarcomas. Sections of sarcomas were examined immunohistochemically by using an anti–TRAIL Ab. (A) Representative sections of leiomyosarcomas at low (LM 1) and high vascularity (LM 2) are shown. Stainings with anti–CD31 Ab to detect endothelial cells and vascular structures, and hematoxylin and eosin (H&E) are also shown. Original magnification, ×20. (B) Liposarcoma (panel a, H&E staining, ×40) showing TRAIL expression in malignant neoplastic cells (arrowhead in panel b; ×40), tumor-infiltrating lymphocytes (panel c; ×40), and plasma cells (arrowhead in panel c; ×100). (C) Kaposi's sarcoma showing diffuse expression of TRAIL. Original magnification, ×40.*

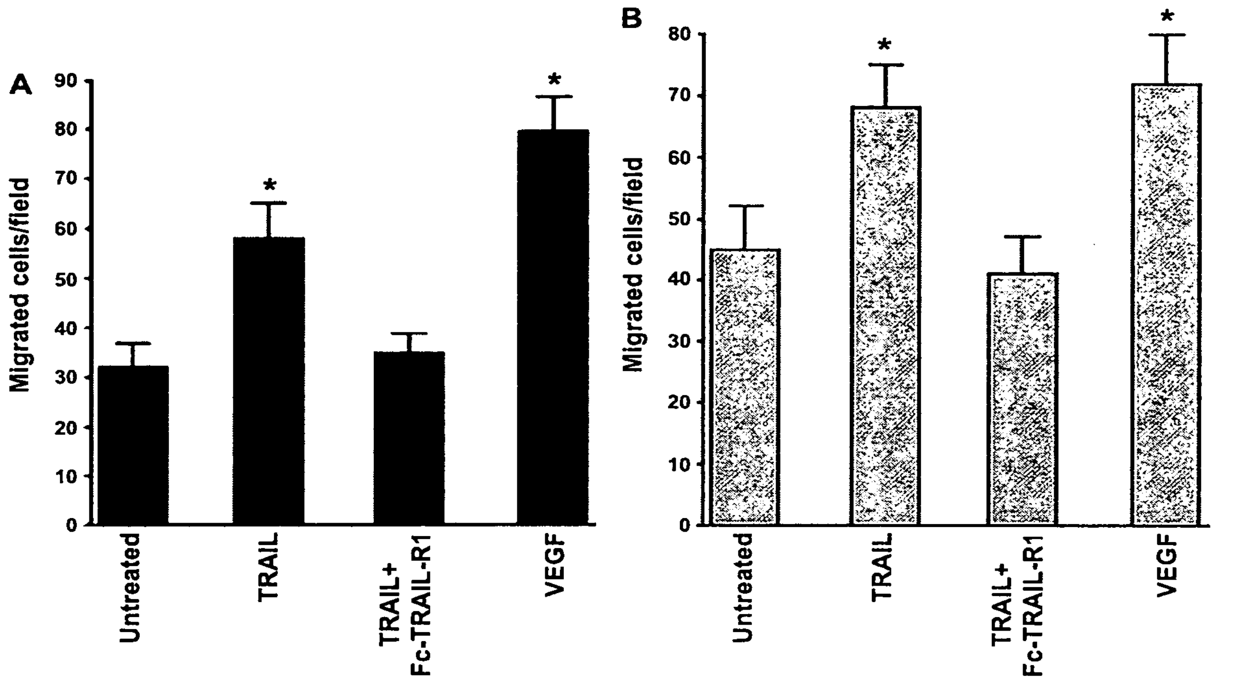


Figure 2. *Effect of TRAIL on HUVEC migration and invasion. Cell migration (A) and cell invasion (B) assays were performed in 24-well Transwell plates, as described in Materials and Methods section. Endothelial cells were seeded in the upper compartments, whereas TRAIL or VEGF was added in the lower compartments. For neutralization experiments, TRAIL was preincubated with TRAIL-R-Fc chimera. Cells migrated through the gelatinized membranes and ECM-coated membranes were counted after 4 hours (A) and 48 (B) hours, respectively. Data are expressed as the number of migrated cells in 10 high-power fields and are mean ± SD of results from four experiments each performed in triplicate. *P < .01 compared to untreated cells.*

angiogenesis coculture assay, which appeared significantly heterogeneous, consisting of both short and long interconnecting tubules that more closely resembled capillaries (Figure 4). In this assay, vessels develop where they are well protected (e.g., between layers of fibroblasts) and morphogenetic processes of tubule formation occur in HUVECs completely surrounded by stromal cells, as *in vivo*. The ability of fibroblasts to support tubule formation has been attributed to their capacity of producing considerable quantities of collagen, fibronectin, and other matrix molecules [19].

To rule out the possibility that the angiogenic activity of TRAIL might be mediated by upregulation of endogenous VEGF, the production of VEGF released in the culture medium was analyzed by enzyme-linked immunosorbent assay (ELISA) in both untreated and TRAIL-treated cultures. In HUVECs, VEGF was not detected in the culture supernatants of both untreated and TRAIL-treated cultures analyzed up to 72 hours (data not shown). In angiogenesis coculture assay, VEGF was endogenously produced, but the levels were similar in untreated (256 ± 36 pg/ml) and TRAIL-treated (240 ± 45 pg/ml) cultures.

We next performed a quantitative analysis by calculating both the total length of tubes and the number of capillary connections per field in cocultures left untreated or exposed to VEGF, TRAIL, and combination of the two cytokines (10 ng/ml each). As shown in Figure 5, the basal formation of capillary-like structures was significantly ($P < .01$) increased after stimulation with either TRAIL or VEGF, and TRAIL-R1-Fc chimeric protein significantly ($P < .01$) inhibited TRAIL-induced total tube length and capillary connections. Moreover, preincubation of TRAIL with 5 μg/ml polymyxin B, which complexes and inactivates endotoxin, did not abrogate the angiogenetic activity of TRAIL, further indicating that these responses to TRAIL are specific. However, the simultaneous addition of TRAIL + VEGF did not show any additive or synergistic effect, and it was not statistically different from VEGF alone (Figure 5).

TRAIL Potentiates VEGF-Induced ERK1/2 But Not p38/MAPK Phosphorylation

The findings illustrated above suggest that TRAIL and VEGF might compete for the same intracellular signal transduction pathways. Therefore, in the next group of experiments, we have investigated the effect of TRAIL and VEGF, used alone or in combination, on mitogen-activated protein (MAP) kinase family members, which have been involved in different aspects of angiogenesis. Although ERK1 and ERK2 are strongly activated on stimulation of cells with mitogens [11], p38/MAPK plays a complex role in angiogenesis by promoting cell migration and inhibiting endothelial cell survival [21–24]. After exposure to either TRAIL or VEGF, induction of phospho-ERK1/2 was observed starting at 5 minutes of treatment (Figure 6*A*). Remarkably, the

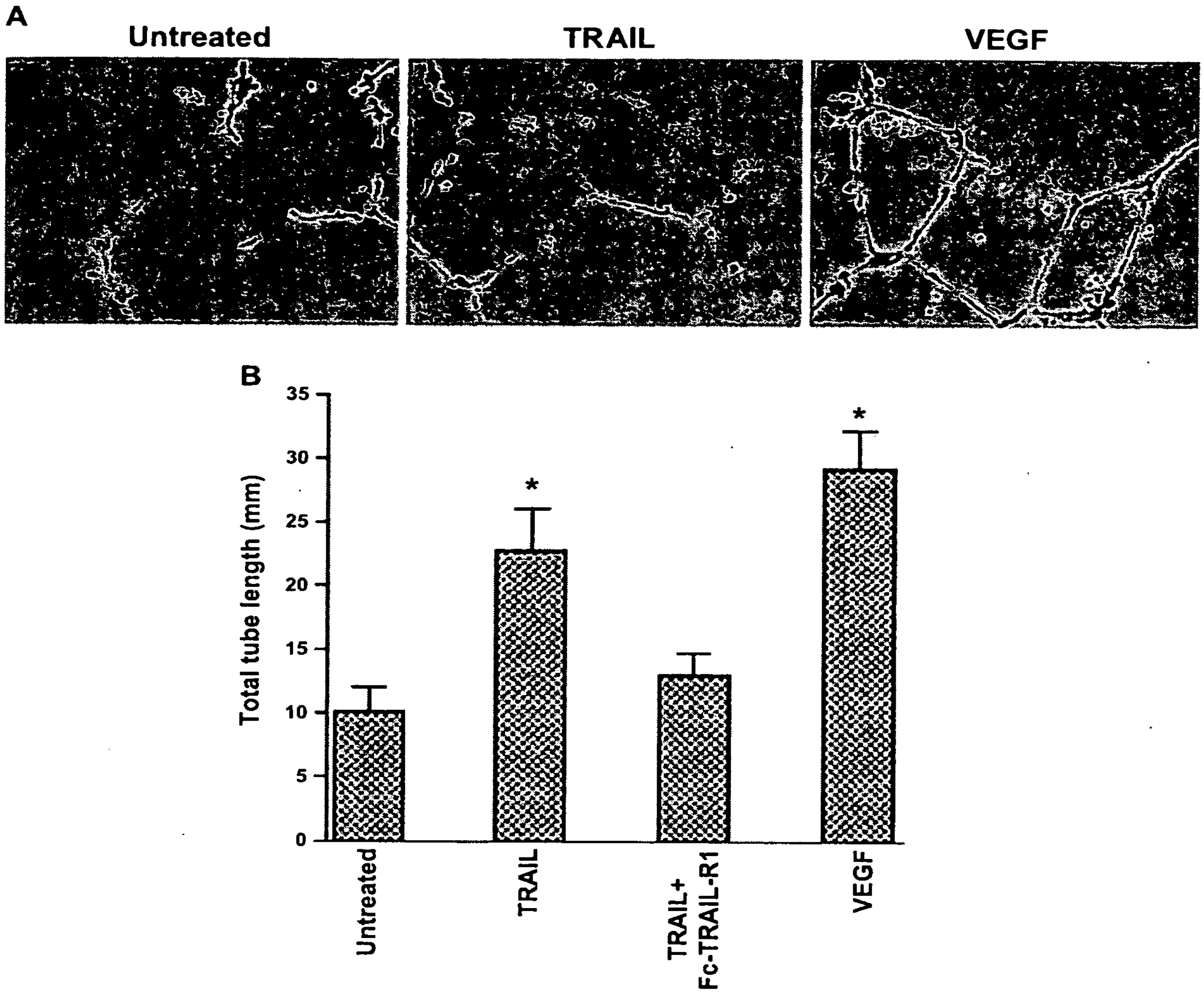


Figure 3. *Effect of TRAIL on in vitro tube formation. HUVECs were seeded into 24 wells containing 3D Matrigel in the absence and presence of TRAIL ± TRAIL-R-Fc or VEGF. (A) Photographs (×40) were taken at 48 hours of cultures. Representative images of at least five experiments with similar results are shown. (B) Five to seven random fields were photographed and recorded, and tube length was quantified by measuring the total cell projection length and individual tubular structure. Tubular length per field is reported as mean ± SD. *P < .01 compared to untreated cells.*

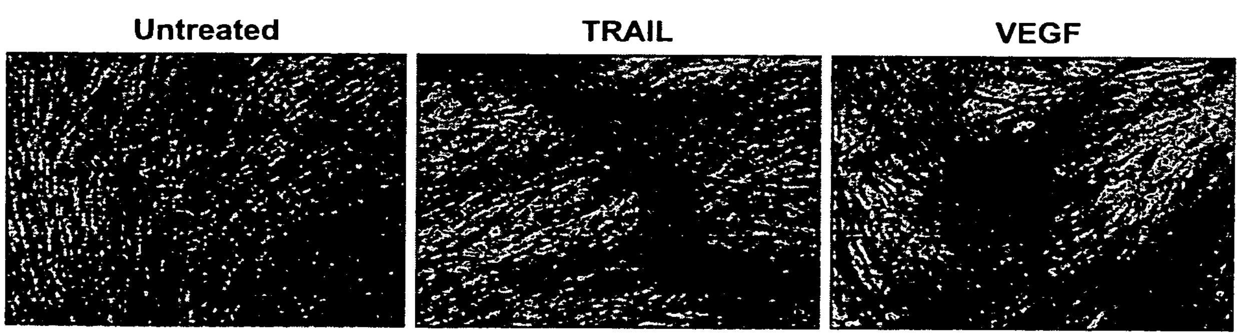


Figure 4. *Effect of TRAIL on the capillary-like network in a coculture angiogenesis assay. Formation of capillary-like structures of HUVECs cultured with matrix-producing cell types was analyzed after 12 days of culture treatment as indicated. Representative example of the morphology endothelial structures was detected after staining with anti–CD31 Ab in the in vitro angiogenesis assay. Rare capillary structures were observed in the angiogenesis assay left untreated, whereas both TRAIL and VEGF induced a diffuse network of capillary structures. Original magnification, ×10.*

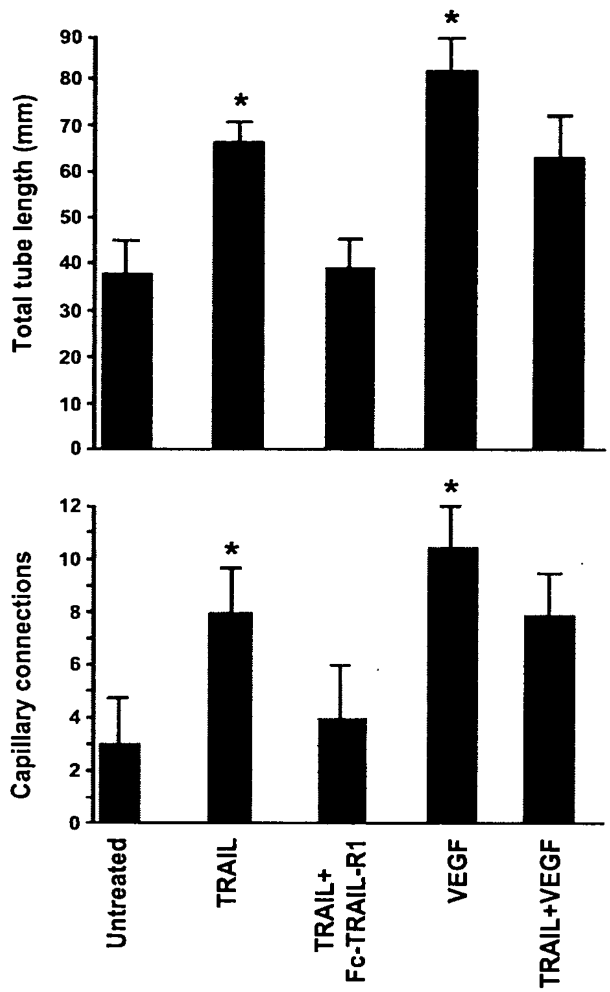


Figure 5. *Lack of additive effect of TRAIL + VEGF on the capillary-like network in coculture angiogenesis assay. Cultures treated as indicated were observed after 12 days and results were recorded for quantitative analysis of the total length of tubes per field and the number of capillary connections per field. Data are expressed as mean ± SD of results from at least five experiments each performed in duplicate. *$P < .01$ compared to untreated cells.*

simultaneous addition of TRAIL + VEGF resulted in a prolonged activation of ERK phosphorylation with respect to each cytokine used alone (Figure 6*A*). However, although TRAIL was unable to induce p38 phosphorylation, it decreased somewhat the VEGF-induced phosphorylation of p38/MAPK (Figure 6*B*). Consistent with a key role of the ERK pathway in endothelial proliferation, thymidine incorporation assay showed that the combination of VEGF + TRAIL showed an additive effect ($P < .01$) with respect to VEGF or TRAIL used alone (Table 1). Preincubation with the cell-permeable PD098059 compound (20 μM), a commonly used inhibitor of the ERK pathway, completely inhibited thymidine uptake induced by any cytokine combination (Table 1), clearly confirming that the activity of ERK is required for TRAIL- and VEGF-induced mitogenesis.

Moreover, PD098059 treatment strongly suppressed the total tube length and number of tube interconnections in control, TRAIL-treated, and VEGF-treated cocultures (Figure 7), underscoring the key role of ERK pathway in the whole process of tube formation evaluated in the angiogenesis assay.

Discussion

The growth of microvessels is an integral component of tissue remodeling during a variety of normal and pathological events, such as the female reproductive cycle, fetal development, wound healing, inflammation, diabetic retinopathy, and tumor progression [13,14]. The way in which vessels form is being intensively studied because this complex morphogenetic process is very important in medicine. Although a true understanding of the morphogenetic processes involved in tubule formation is still lacking, all these angiogenic events are orchestrated by a network of extracellular factors, including several classes of cytokines, ECM, and integrins, and by their cognate receptors. Several cytokines have been involved in angiogenesis and, in particular, in tumor-associated angiogenesis [13,14]. Besides its involvement in vascular development, VEGF has been demonstrated to play a key role in both physiologic and tumor angiogenesis in adult mammals [25].

It has also been shown in previous studies that some angiogenic regulators belong to the TNF family [26]. Ligands of this family trigger biological activities by binding and signaling through their corresponding receptors in the TNF receptor family. The majority of the TNF family members mediate host defense, inflammation, and immunological regulation, but some of these ligands also regulate endothelial cell functions [26]. For instance, it has been demonstrated that TNF-α modulates endothelial cell behavior; however, its effects are complex. TNF-α inhibits endothelial cell growth yet induces capillary tube formation *in vitro* [27,28]. It also can be antiangiogenic in the context of solid tumors, or angiogenic in corneal settings *in vivo* [27–29]. In a recent study, we have demonstrated that TRAIL functions as an anti-apoptotic factor for endothelial cells [7], and we have hypothesized that TRAIL may contribute to endothelial cell integrity by acting as a survival factor for newly formed blood vessels.

In this study, we have demonstrated for the first time that TRAIL induces a proangiogenic phenotype in human endothelial cells. This phenotype includes both early (increase in migration, invasion, and proliferation) and late (differentiation into vascular cords) angiogenic events. More importantly, TRAIL is angiogenic in a variety of *in vitro* and *in vivo* assays to a degree comparable to VEGF. However, the interplay between TRAIL and VEGF appears rather complex, with TRAIL unable to potentiate VEGF activity in most assays. In fact, TRAIL increased VEGF-induced ERK phosphorylation and HUVEC proliferation; however, TRAIL did not potentiate VEGF-induced p38/MAPK phosphorylation or capillary formation in the

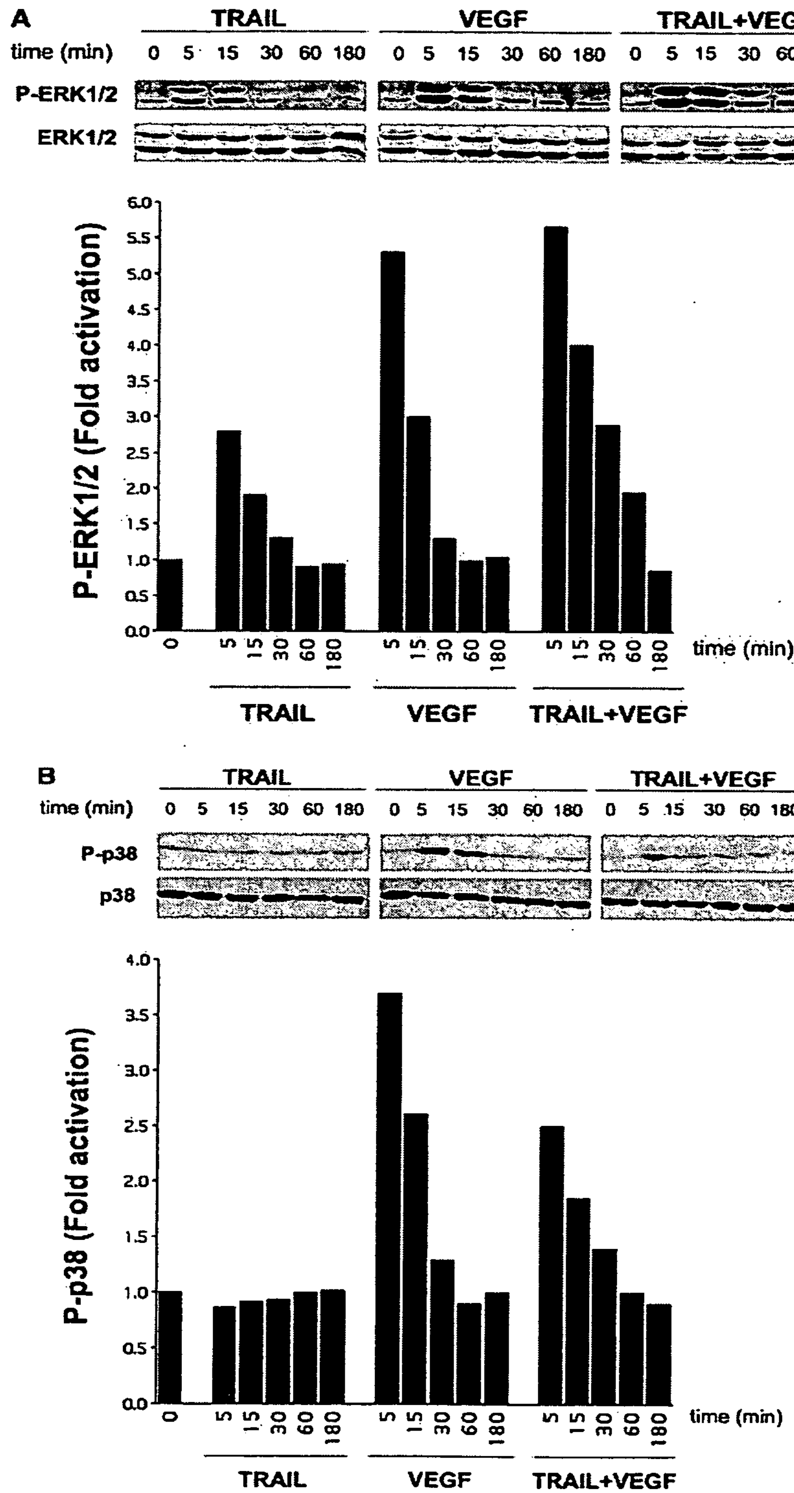


Figure 6. *Phosphorylation of ERK1/2 and p38/MAPK in response to TRAIL and VEGF. Quiescent HUVECs were stimulated with either TRAIL, VEGF, or VEGF + TRAIL for 0 to 180 minutes. Cell lysates were analyzed for ERK1/2 (A) and p38/MAPK (B) activation by Western blot analysis of total and phosphorylated (P) proteins using specific antibodies. Protein bands were quantified by densitometry, and levels of P-ERK1/2 and P-p38 were calculated for each time point, after normalization to ERK1/2 and to p38/MAPK, respectively. Unstimulated basal expression was set as unity. Results are representative of four separate experiments.*

coculture assay. Of note, we have also demonstrated that a clear-cut expression of TRAIL was observed in highly vascularized soft tissue sarcomas. At the moment, we have analyzed a small number of sarcomas; experiments aimed to analyze a high number of tumors and a wide variety of tumors, with the aim to quantitatively evaluate potential correlations between microvessel density and the levels of TRAIL expression, are ongoing.

Besides malignant sarcoma cells, TRAIL was expressed also by tumor-infiltrating lymphocytes and plasma cells. Similar findings were obtained by previous authors describing that TRAIL was expressed by tumor-infiltrating lymphocytes as well as by metastatic gastric cancer cells [30]. Although the most frequent hypothesis to explain the expression of TRAIL by cancer cells is a strategy of immune evasion by which TRAIL-positive malignant cells counterattack against activated T lymphocytes [30,31], our data suggest the alternative—not mutually exclusive—hypothesis that TRAIL expression by cancer cells, and, perhaps also by infiltrating lymphocytes, may play a key role in tumor angiogenesis. Consistent with this hypothesis, it has been shown that mouse BALB/c mammary adenocarcinoma cells engineered to express human TRAIL on their membrane grow faster than the parental cell line in both syngeneic and allogeneic mice [32]. In this respect, it has been clearly established that any significant increase in tumor mass must be preceded by an increase in the vascular supply to deliver nutrients and oxygen to the tumor. The ability of a tumor to induce angiogenesis represents an essential step for tumor growth beyond 2 to 3 mm [13]. Consistent with a potentially important role of TRAIL in tumor angiogenesis not confined to soft tissue sarcomas, it has been demonstrated that all primary astrocytic brain tumors analyzed by immunohistochemistry, including astrocytomas and glioblastomas, express TRAIL protein *in vivo* [33,34]. Similarly, TRAIL is expressed by a subset of lymphomas [35], malignant plasmocytoma cells [36], and ovarian carcinomas [37], in which TRAIL expression has been correlated to the degree of malignancy. Therefore, although recombinant TRAIL protein offers great promise as a cancer therapeutic [38], our current demonstration that TRAIL exerts a potent proangiogenic effect adds a cautionary note to the prolonged treatment of cancer patients with pharmacological concentrations of recombinant TRAIL protein or TRAIL-expressing vectors.

Table 1. Thymidine Incorporation Assay in HUVEC Cultures.

	Vehicle	PD098059
Untreated	2700 ± 350	570 ± 80
TRAIL	3850 ± 600	588 ± 98
VEGF	4200 ± 470	550 ± 90
VEGF + TRAIL	5400 ± 550	600 ± 105

Values are expressed as counts per minute (cpm) per well and are mean±SD of three separate experiments.

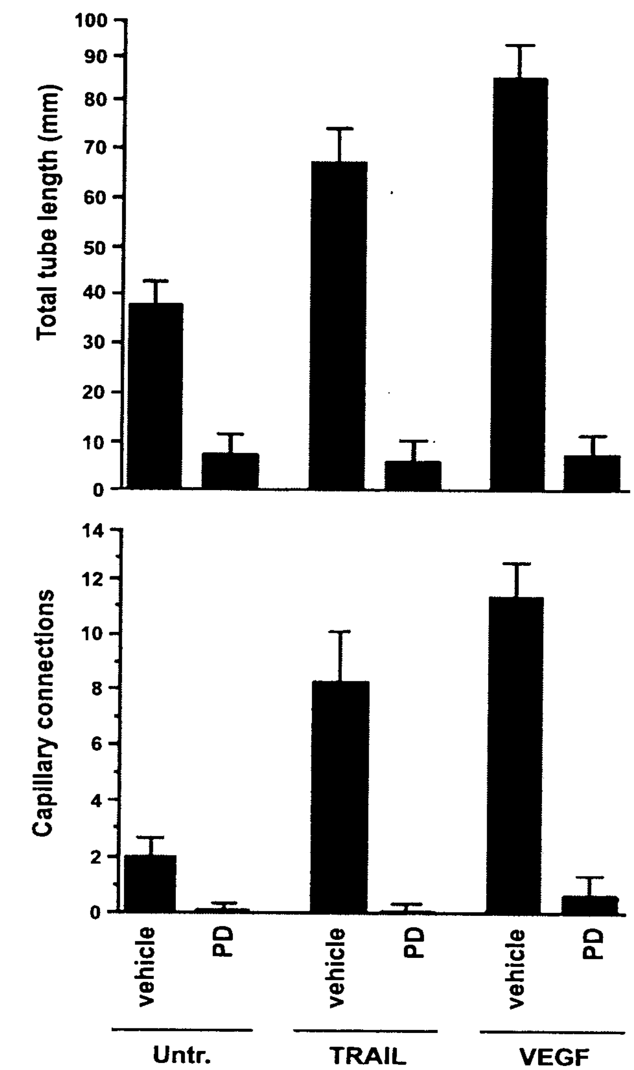


Figure 7. *Role of ERK pathway in capillary-like network formation in the coculture angiogenesis assay. Cultures treated as indicated were observed after 12 days and results were recorded for quantitative analysis of the total length of tubes per field and the number of capillary connections per field. PD, PD98059. Data are expressed as mean ± SD of results from at least five experiments each performed in duplicate.*

References

[1] Ashkenazi A (2002). Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* **2**, 420–430.

[2] Mariani SM and Krammer PH (1998). Differential regulation of TRAIL and CD95 ligand in transformed cells of the T and B lymphocyte lineage. *Eur J Immunol* **28**, 973–982.

[3] Walczak H, Miller RE, Ariail K, Gliniak TS, Griffith B, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC, and Lynch DH (1999). Tumoricidal activity of tumor necrosis factor–related apoptosis-inducing ligand *in vivo*. *Nat Med* **5**, 157–163.

[4] Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussi J, Koeppen H, Shahrokh Z, and Schwall RH (1999). Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* **104**, 155–162.

[5] Sheridan JP, Marsters SA, Pitti PM, Gurney A, Skubatch M, Baldwin D,

Yagita H, Gray CL, Baker K, Wood WI, Goddard AD, Godowski P, and Ashkenazi A (1997). Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* 277, 818–822.

[6] Zhang XD, Nguyen T, Thomas WD, Sanders JE, and Hersey P (2000). Mechanisms of resistance of normal cells to TRAIL induced apoptosis vary between different cell types. *FEBS Lett* 482, 193–199.

[7] Secchiero P, Gonelli A, Carnevale E, Milani D, Pandolfi A, Zella D, and Zauli G (2003). TRAIL promotes the survival and proliferation of primary human vascular endothelial cells by activating the Akt and ERK pathways. *Circulation* 107, 2250–2256.

[8] Zauli G, Pandolfi A, Gonelli A, Di Pietro R, Guarnieri S, Ciabattoni G, Rana R, Vitale M, and Secchiero P (2003). Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) sequentially upregulates nitric oxide and prostanoid production in primary human endothelial cells. *Circ Res* 92, 732–740.

[9] Gochuico BR, Zhang J, Ma BJ, Marshak-Rothstein A, and Fine A (2000). TRAIL expression in vascular smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 278, L1045–L1050.

[10] Chapman HA, Riese RJ, and Shi GP (1997). Emerging roles for cysteine proteases in human biology. *Annu Rev Physiol* 59, 63–88.

[11] Yu Y and Sato JD (1999). MAP kinases, phosphatidylinositol 3-kinase, and p70 S6 kinase mediate the mitogenic response of endothelial cells to vascular endothelial growth factor. *J Cell Physiol* 178, 235–246.

[12] Morales-Ruiz M, Fulton G, Sowa G, Languino LR, Fujio Y, Walsh K, and Sessa WC (2000). Vascular endothelial growth factor–stimulated actin reorganization and migration of endothelial cells is regulated via the serine/threonine kinase Akt. *Circ Res* 86, 892–896.

[13] Folkman J (2001). Angiogenesis-dependent diseases. *Semin Oncol* 28, 536–542.

[14] Carmeliet P (2003). Angiogenesis in health and disease. *Nat Med* 9, 653–660.

[15] Dirix LY, Vermeulen P, De Wever I, and Van Oosterom AT (1997). Soft tissue sarcoma in adults. *Curr Opin Oncol* 9, 348–359.

[16] Weidner N (1995). Intratumor microvessel density as a prognostic factor in cancer. *Am J Pathol* 147, 9–19.

[17] Vermeulen PB, Gasparini G, Fox SB, Toi M, Martin L, McP Culloch F, Pezzella F, Viale G, Weidner N, Harris AL, and Dirix LY (1996). Quantification of angiogenesis in solid human tumor: an international consensus in the methodology and criteria of evaluation. *Eur J Cancer* 32A, 2474–2484.

[18] Passaniti A, Tylor RM, Pili R, Guo Y, Long PV, Haney A, Pauly RR, Grant DS, and Martin GR (1992). A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin and fibroblast growth factor. *Lab Invest* 67, 519–528.

[19] Donovan D, Brown NJ, Bishop ET, and Lewis CE (2001). Comparison of three *in vitro* human angiogenesis assays with capillaries formed *in vivo*. *Angiogenesis* 4, 113–121.

[20] Mentzel T, Brown LF, Dvorak HF, Kuhnen C, Stiller KJ, Katenkamp D, and Fletcher CDM (2001). The association between tumour progression and vascularity in myxofibrosarcoma and myxoid/round cell liposarcoma. *Virchows Arch* 438, 13–22.

[21] Liabakk NB, Sundan A, Torp S, Aukrust P, Froland SS, and Espevik T (2002). Development, characterization and use of monoclonal antibodies against sTRAIL: measurement of sTRAIL by ELISA. *J Immunol Methods* 259, 119–128.

[22] Rousseau S, Houle F, Kotanides H, Witte L, Waltenberger J, Landry J, and Huot J (2000). Vascular endothelial growth factor (VEGF)–driven actin-based motility is mediated by VEGFR2 and requires concerted activation of stress-activated protein kinase 2 (SAPK2/p38) and geldanamycin-sensitive phosphorylation of focal adhesion kinase. *J Biol Chem* 275, 10661–10672.

[23] Gratton JP, Morales-Ruitz M, Kureishi Y, Fulton D, Walsh K, and Sessa W (2001). Akt down-regulation of p38 signaling provides a novel mechanism of vascular endothelial growth factor–mediated cytoprotection in endothelial cells. *J Biol Chem* 276, 30359–30365.

[24] Matsumoto T, Turesson I, Book M, Gerwins P, and Claesson-Welsh L (2002). p38 MAP kinase negatively regulates endothelial cell survival, proliferation and differentiation in FGF-2–stimulated angiogenesis. *J Cell Biol* 156, 149–160.

[25] Ferrara N, Gerber HP, and LeCouter J (2003). The biology of VEGF and its receptors. *Nat Med* 9, 669–676.

[26] Locksley RM, Killeen N, and Lenardo MJ (2001). The TNF and TNF receptor superfamilies. Integrating mammalian biology. *Cell* 104, 487–501.

[27] Frater-Schroder M, Risau W, Hallmann R, Gautschi P, and Bohlen P (1987). Tumor necrosis factor type α, a potent inhibitor of endothelial cell growth *in vitro*, is angiogenic *in vivo*. *Proc Natl Acad Sci USA* 84, 5277–5281.

[28] Yoshida S, Ono M, Shono T, Izumi H, Ishibashi T, Suzuki H, and Kuwano M (1997). Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha–dependent angiogenesis. *Mol Cell Biol* 17, 4015–4023.

[29] Ruegg C, Yilmaz A, Bieler G, Bamat J, Chaubert P, and Lejeune FJ (1998). Evidence for the involvement of endothelial cell integrin alphaV-beta3 in the disruption of the tumor vasculature induced by TNF and IFN-gamma. *Nat Med* 4, 408–414.

[30] Koyama S, Koike N, and Adachi S (2002). Expression of TNF-related apoptosis-inducing ligand (TRAIL) and its receptors in gastric carcinoma and tumor-infiltrating lymphocytes: a possible mechanism of immune evasion of the tumor. *J Cancer Res Clin Oncol* 128, 73–79.

[31] Takeda K, Smyth MJ, Cretney E, Hayakawa Y, Kayagaki N, Yagita H, and Okumura K (2002). Critical role for tumor necrosis factor–related apoptosis-inducing ligand in immune surveillance against tumor development. *J Exp Med* 195, 161–169.

[32] Giovarelli M, Musiani P, Garotta G, Ebner R, Di Carlo E, Kim Y, Cappello P, Rigamonti L, Bernabei P, Novelli F, Modesti A, Coletti A, Ferrie AK, Lollini PL, Ruben S, Sakcedi T, and Forni G. A "Stealth Effect": adenocarcinoma cells engineered to express TRAIL elude tumor-specific and allogeneic T cell reactions. *J Immunol* 163, 4886–4893.

[33] Rieger J, Ohgaki H, Kleihues P, and Weller M (1999). Human astrocytic brain tumors express APO2L/TRAIL. *Acta Neuropathol* 97, 1–4.

[34] Frank S, Kohler U, Schackert G, and Schackert HK (1999). Expression of TRAIL and its receptors in human brain tumors. *Biochem Biophys Res Commun* 257, 454–459.

[35] Zhao S, Asgary Z, Wang Y, Goodwin R, Andreeff M, and Younes A (1999). Functional expression of TRAIL by lymphoid and myeloid tumor cells. *Br J Haematol* 106, 827–832.

[36] Silvestris F, Cafforio P, Tucci M, and Dammacco F (2002). Negative regulation of erythroblast maturation by Fas-L(+)/TRAIL(+) highly malignant plasma cells: a major pathogenetic mechanism of anemia in multiple myeloma. *Blood* 99, 1305–1313.

[37] Lancaster JM, Sayer R, Blanchette C, Calingaert B, Whitaker R, Schildraut J, Marks J, and Berchuck A (2003). High expression of tumor necrosis factor–related apoptosis-inducing ligand is associated with favorable ovarian cancer survival. *Clin Cancer Res* 9, 762–767.

[38] Smyth MJ, Takeda K, Hayakawa Y, Peschon JJ, van den Brink MRM, and Yagita H (2003). Nature's TRAIL—on a path to cancer immunotherapy. *Immunity* 18, 1–6.

Exhibit B

MOLECULAR AND CELLULAR BIOLOGY, Apr. 2002, p. 2830–2841 Vol. 22, No. 8
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.8.2830–2841.2002

Activated Notch4 Inhibits Angiogenesis: Role of β1-Integrin Activation

Kevin G. Leong,[1,2] Xiaolong Hu,[3] Linheng Li,[4] Michela Noseda,[2,3] Bruno Larrivée,[1,2] Christopher Hull,[2,3] Leroy Hood,[5] Fred Wong,[2,3] and Aly Karsan[1,2,3]*

Departments of Experimental Medicine[1] and Pathology and Laboratory Medicine,[3] University of British Columbia and British Columbia Cancer Agency, and Department of Medical Biophysics, British Columbia Cancer Agency,[2] Vancouver, British Columbia V5Z 1L3, Canada; Stem Cell Research Laboratory, Stowers Institute for Medical Research, Kansas City, Missouri 64110[4]; and Institute for Systems Biology, Seattle, Washington 98105[5]

Received 22 May 2001/Returned for modification 7 July 2001/Accepted 9 January 2002

Notch4 is a member of the Notch family of transmembrane receptors that is expressed primarily on endothelial cells. Activation of Notch in various cell systems has been shown to regulate cell fate decisions. The sprouting of endothelial cells from microvessels, or angiogenesis, involves the modulation of the endothelial cell phenotype. Based on the function of other Notch family members and the expression pattern of Notch4, we postulated that Notch4 activation would modulate angiogenesis. Using an in vitro endothelial-sprouting assay, we show that expression of constitutively active Notch4 in human dermal microvascular endothelial cells (HMEC-1) inhibits endothelial sprouting. We also show that activated Notch4 inhibits vascular endothelial growth factor (VEGF)-induced angiogenesis in the chick chorioallantoic membrane in vivo. Activated Notch4 does not inhibit HMEC-1 proliferation or migration through fibrinogen. However, migration through collagen is inhibited. Our data show that Notch4 cells exhibit increased β1-integrin-mediated adhesion to collagen. HMEC-1 expressing activated Notch4 do not have increased surface expression of β1-integrins. Rather, we demonstrate that Notch4-expressing cells display β1-integrin in an active, high-affinity conformation. Furthermore, using function-activating β1-integrin antibodies, we demonstrate that activation of β1-integrins is sufficient to inhibit VEGF-induced endothelial sprouting in vitro and angiogenesis in vivo. Our findings suggest that constitutive Notch4 activation in endothelial cells inhibits angiogenesis in part by promoting β1-integrin-mediated adhesion to the underlying matrix.

Angiogenesis, the formation of new blood vessels from existing vessels, is a complex process requiring modulation of multiple endothelial cell functions (3, 30, 62). The formation of capillary sprouts from the existing microvasculature occurs secondary to an inciting stimulus that results in increased vascular permeability, accumulation of extravascular fibrin, and local proteolytic degradation of the basement membrane (20, 59). The endothelial cells overlying the disrupted region become activated, change shape, and extend elongated processes into the surrounding tissue (20, 59). Directed migration toward the angiogenic stimulus results in the formation of a column of endothelial cells (3, 30, 62). Just proximal to the migrating tip of the column is a region of proliferating cells (3, 30). These proliferating endothelial cells cause an increase in the length of the sprout. Proximal to the proliferative zone, the endothelial cells undergo another shape change, adhere tightly to each other, and begin to form a lumen (3, 30). Secondary sprouting from the migrating tip results in a capillary plexus, and the fusion of individual sprouts at their tips closes the loop and circulates blood into the vascularized area (3, 30, 62). Throughout this process the function and expression of various adhesion proteins, including those of the integrin family, are tightly regulated (5, 15). Several growth factors and cytokines are known to stimulate angiogenesis, the best-studied of which are vascular endothelial growth factor (VEGF), and fibroblast growth factor 2 (FGF-2; basic FGF) (20, 60).

During development, equipotential cells choose between alternative cell fates. Interactions between the Notch transmembrane receptor and its various ligands on adjacent cells can determine cell fate (2, 51). Notch is also involved in signaling between heterotypic cells to modulate differentiation (2, 51). The importance of Notch in mammalian differentiation is highlighted by several mutations responsible for human disease (22, 37, 48). Engagement of Notch by a ligand results in cleavage of the receptor within or close to the plasma membrane, with subsequent translocation of the C-terminal intracellular domain (NotchIC) to the nucleus (64, 71). Because activation of Notch requires ligand-dependent cleavage of the intracellular domain, enforced expression of NotchIC results in a constitutively active form of the receptor (29, 61). Enforced expression of the truncated intracellular domain of Notch proteins inhibits differentiation pathways in several models but is required for differentiation in other systems (6, 27, 41).

Four mammalian Notch homologues have been identified to date (Notch1 to -4) (47, 51, 74). Recently, the full-length form of Notch4 was cloned from mice and humans (47, 74). Notch4 is evolutionarily distant from the other members of the Notch family (47). Distinct structural features of Notch4 include fewer epidermal growth factor-like repeats and an intracellular domain significantly shorter than those of other Notch members (74). Of interest to us is that Notch4 is primarily expressed on the endothelium and the endocardium (47, 68, 74).

Given that Notch4 is primarily expressed on endothelial cells, we postulated that Notch4 may be involved in regulating

* Corresponding author. Mailing address: Department of Medical Biophysics, British Columbia Cancer Research Centre, 601 West 10th Ave., Vancouver, British Columbia, Canada V5Z 1L3. Phone: (604) 877-6248. Fax: (604) 877-6002. E-mail: akarsan@bccancer.bc.ca.

angiogenesis. To answer this question, we expressed the truncated, constitutively active intracellular domain of Notch4 (Notch4IC) in endothelial cells. Our studies indicate that activated Notch4 inhibits the sprouting of human dermal microvascular endothelial cells (HMEC-1) in vitro and angiogenesis in the chick chorioallantoic membrane (CAM) in vivo. Activated Notch4 does not inhibit proliferation of HMEC-1, nor does it inhibit their migration through fibrinogen toward angiogenic factors FGF-2 and VEGF. However, activated Notch4 does inhibit migration through collagen. We demonstrate that the decreased sprouting of Notch4IC cells from collagen-coated beads is due in part to enhanced β1-integrin-mediated adhesion to collagen. Although endothelial cells expressing Notch4IC do not show increased surface expression of β1-integrins, we show that the β1-integrins are in a high-affinity, active conformation. We also show that activation of β1-integrins with function-activating β1-integrin monoclonal antibodies, independent of Notch4 expression, is sufficient to inhibit endothelial sprouting in vitro and angiogenesis in vivo. Thus, our results suggest that Notch4 activation in endothelial cells in vivo may inhibit angiogenesis in part by promoting β1-integrin-mediated adhesion to the underlying matrix.

MATERIALS AND METHODS

Cell culture. The HMEC-1 (referred to hereafter as HMEC) line (1) was provided by the Centers for Disease Control and Prevention (Atlanta, Ga.). HMEC lines were cultured in MCDB medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 10 μg of epidermal growth factor/ml, and 100 U each of penicillin and streptomycin/ml. The avian retroviral packaging cell line Q2bn (gift from K. McNagny, University of British Columbia) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% non-heat-inactivated FCS, 2.5% non-heat-inactivated chicken serum, 60 μg of conalbumin/ml, 50 μM β-mercaptoethanol, 2 mM glutamine, and 100 U each of penicillin and streptomycin/ml. All cells were maintained at 37°C in 5% CO_2.

Gene transfer. HMEC-Notch4IC and HMEC-LNCX were constructed by retroviral transduction of cDNA encoding a C-terminal hemagglutinin (HA)-tagged human Notch4 (amino acids 1476 to 2003) or of the empty pLNCX vector control, respectively (47). The method of transduction has previously been described (39). Stable HMEC lines were obtained by selection in 300 μg of G-418 (Gibco)/ml. Polyclonal HMEC lines were used to avoid artifacts due to the retroviral integration site. Chicken retroviral expression vectors were constructed by inserting C-terminal HA-tagged human Notch4IC cDNA into the avian retroviral vector CK (gift from N. Boudreau, University of California, San Francisco, and M. Bissell, University of California, Berkeley). Both CK-Notch4IC and the empty vector were transiently transfected into Q2bn cells with Fugene 6 transfection reagent (Boehringer Mannheim) to generate producer lines.

Immunoblotting and immunofluorescence. For immunoblotting, total cellular extracts were prepared from HMEC or Q2bn lines by lysing 10^5 cells in a solution containing 50 mM Tris, 150 mM NaCl, 2% Triton X-100, 10 μg of soybean trypsin inhibitor/ml, and 200 μM phenylmethylsulfonyl fluoride and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting as previously described (19).

For immunofluorescence, HMEC lines (5×10^4 cells) were cultured on coverslips for 48 h, fixed, and permeabilized in cold methanol for 5 min. Nonspecific binding was blocked by incubation with phosphate-buffered saline (PBS) containing 5% goat serum and 0.1% Tween 20. Following incubation with a primary antibody (rabbit anti-HA polyclonal antibody; 1:100 dilution) for 1 h and a secondary antibody (Texas red-conjugated goat anti-rabbit immunoglobulin G [IgG]; 1:200 dilution) for 30 min, coverslips were mounted on glass slides with an antifading solution (FluoroGuard antifade reagent; Bio-Rad) containing 100 ng of Hoechst 33258 (Sigma)/ml to stain the nuclei. Immunofluorescence was examined using an Axioplan 2 imaging microscope (Zeiss), and images were captured with a DVC-1310M digital camera (Digital Video Camera Company).

Endothelial-sprouting assay. Endothelial sprouting was assessed by a modification of the method of Nehls and Drenckhahn (53). Briefly, microcarrier beads coated with gelatin (Cytodex 3; Sigma) or positively charged, cross-linked dextran (Cytodex 2; Sigma) were seeded with HMEC lines. When the cells reached confluence on the beads, equal numbers of HMEC-coated beads were embedded in fibrin gels in 96-well plates. For preparation of fibrin gels, bovine fibrinogen was dissolved in PBS at a concentration of 2.5 mg/ml. Aprotinin was added at a concentration of 0.05 mg/ml, and the solution was filtered through a 0.22-μm-pore-size filter. Fibrinogen solution was supplemented with FGF-2 (15 ng/ml) or VEGF (15 ng/ml). As a control, fibrinogen solution without angiogenic factor was used. Following transfer of the fibrinogen solution to 96-well plates, HMEC-coated beads were added at a density of 50 beads/well, and clotting was induced by the addition of thrombin (1.2 U/ml). After clotting was complete, gels were equilibrated with MCDB–2% FCS at 37°C. Following 60 min of incubation, the overlying medium was changed for all wells. MCDB–2% FCS, either alone or containing FGF-2 (15 ng/ml) or VEGF (15 ng/ml), was added to the wells. After 3 days of incubation with daily medium changes, the number of capillary-like tubes formed was quantitated by counting the number of tube-like structures per microcarrier bead (sprouts per bead). Only sprouts greater than 150 μm in length and composed of at least three endothelial cells were counted.

For coating Cytodex 2 beads with collagen, beads were resuspended in 1 mg of collagen type I/ml, allowed to dry overnight on petri dishes, and resuspended in PBS. For coating Cytodex 2 beads with antibodies (IgG2a, 1:1,000 dilution [Sigma]; 8A2, 1:1,000 dilution [gift from J. Harlan, University of Washington]; LM534, 1:1,000 dilution [Chemicon]), beads were incubated with antibodies at 37°C for 2 h, washed twice with PBS, and resuspended in PBS. After the antibody-coated beads were incubated with cells for 3 days, the beads were placed in fibrin gels supplemented with the appropriate antibody at 1:1,000 dilution.

CAM assay. Fertilized White Leghorn chicken (*Gallus gallus domesticus*) eggs were incubated at 37°C under conditions of constant humidity. All chicken eggs were handled according to institutional animal care procedures. On embryonic day 6, the developing CAM was separated from the shell by opening a small circular window at the broad end of the egg above the air sac. The embryos were checked for normal development, the window was sealed with Parafilm, and the eggs were returned to the incubator for two more days. On day 8, transfected Q2bn cell lines were trypsinized and washed in PBS, and 3×10^6 cells resuspended in 15 μl of DMEM supplemented with 30 ng of VEGF/ml were placed onto nylon meshes (pore size, 250 μm; Sefar America) on the CAM. The cells distribute throughout the mesh and secrete control virus or virus containing Notch4IC. Meshes treated with vehicle alone (15 μl of DMEM) were used as negative controls, whereas meshes treated with VEGF (30 ng/ml in 15 μl of DMEM) were used as positive controls. Eggs were resealed and returned to the incubator. On day 12, images of the CAMs were captured digitally with an Olympus SZX9 stereomicroscope (Olympus America) equipped with a Spot RT digital imaging system (Diagnostic Instruments). Neovascularization was quantitated for each CAM by counting the number of vessels that entered the mesh area and dividing by the perimeter of the mesh (vessels per millimeter). Northern Eclipse, version 6.0 (Empix Imaging, Inc.), was used for manual vessel counting and mesh perimeter measurements. Following photography, CAMs were harvested and processed for further studies.

For CAMs treated with anti-integrin antibodies, fertilized White Leghorn chicken eggs were prepared as described above. Mouse anti-avian β1-integrin antibodies TASC (9D11; function-activating β1-integrin antibody; gift of L. F. Reichardt, University of California, San Francisco), V2E9 (non-function-modifying β1-integrin antibody; Developmental Studies Hybridoma Bank, University of Iowa), and W1B10 (function-blocking β1-integrin antibody; Sigma) were prepared at 10 μg/ml in PBS supplemented with 30 ng of VEGF/ml. On day 8, 20 μl of each antibody preparation was loaded onto 2-mm^3 gelatin sponges (Gelfoam; Pharmacia Upjohn), which were then placed on the surface of the developing CAM. Sponges containing vehicle alone (20 μl of PBS) were used as negative controls, whereas sponges containing 20 μl of VEGF at 30 ng/ml in PBS were used as positive controls. CAMs were also treated with function-blocking mouse anti-human αvβ3 antibody LM609 (which cross-reacts with avian αvβ3 integrin; Chemicon) prepared at 10 μg/ml in PBS containing 30 ng of VEGF/ml. LM609 has previously been shown to attenuate VEGF-induced angiogenesis in the CAM (23) and thus serves as a positive control for angiogenesis inhibition. Eggs were resealed and returned to the incubator. On day 10, digital images of the CAMs were captured and analyzed for neovascularization as described above.

Immunohistochemistry. CAMs treated with transfected Q2bn cell lines were harvested from day 12 embryos and processed for histological analysis. For hematoxylin and eosin (H&E) staining, CAMs were fixed in formalin overnight at room temperature, dehydrated, and embedded in paraffin. Sections (6 μm thick) were cut and stained with H&E. For immunohistological analysis, CAMs were frozen in Tissue-Tek optimal cutting temperature compound (Somagen) and 10-μm-thick sections were cut and fixed in acetone for 10 min. Sections were

hydrated and incubated in 1.5% hydrogen peroxide solution for 5 min to quench endogenous peroxide activity. Nonspecific binding was blocked by incubation in normal goat serum (1:20 dilution) for 20 min. For von Willebrand factor (vWF) staining, sections were incubated with a primary antibody (1:200 dilution; DAKO) and a biotinylated secondary antibody, followed by an avidin conjugate. For HA staining, sections were incubated with a primary antibody (mouse anti-HA monoclonal antibody, 1:500 dilution) and a secondary antibody (biotinylated goat anti-mouse IgG, 1:200 dilution), followed by peroxidase-conjugated streptavidin (DAKO). All sections were developed with a diaminobenzidine-hydrogen peroxide reaction (Sigma), counterstained with hematoxylin, dehydrated, cleared, and mounted. CAM sections were examined with an Axioplan 2 imaging microscope (Zeiss), and images were captured with a Coolpix 990 digital camera (Nikon).

Proliferation assay. Proliferation of endothelial cells in response to angiogenic factors FGF-2 and VEGF was determined by two methods: (i) neutral red uptake and (ii) flow-cytometric analysis for total DNA content (DAPI [4′,6′-diamidino-2-phenylindole]) and bromodeoxyuridine (BrdU) incorporation. For the neutral red assay (19, 49), confluent plates of HMEC lines were serum starved in MCDB–2% FCS for 48 h and cells were plated in 96-well plates at a density of 5×10^3 cells/well. After 4 h of incubation to allow cells to bind, overlay medium was removed and the cells were treated with MCDB–2% FCS supplemented with FGF-2 (15 ng/ml) or VEGF (15 ng/ml). As a control, cells were treated with MCDB–2% FCS alone. Cells were incubated for 0, 24, 48, and 72 h with daily medium changes. After each time point, wells were emptied and incubated with 100 μl of neutral red dye (0.0025% neutral red in MCDB–2% FCS). Empty wells were also incubated with neutral red dye for background absorbance correction. After 4 h of incubation, wells were aspirated and neutral red dye was solubilized with 100 μl of 1% acetic acid–50% ethanol per well. Absorbance was determined at 570 nm.

For the flow-cytometric analysis of cell cycle distribution, HMEC lines were serum starved in MCDB–2% FCS for 48 h and incubated for an additional 24 h in medium alone (MCDB–2% FCS) or medium supplemented with FGF-2 (15 ng/ml) or VEGF (15 ng/ml). For the last 2 h of incubation, cells were incubated with 10 μM BrdU (Sigma) at 37°C. Cells were harvested by trypsinization and fixed in 70% ethanol at 4°C for 30 min. After being washed in PBS, cells were incubated in 2 M HCl for 30 min to denature DNA, followed by neutralization in serum-free medium. Cells were blocked and permeabilized in 0.5% Triton X-100–4% calf serum and then incubated with an anti-BrdU fluorescein isothiocyanate (FITC)-conjugated antibody (Pharmingen) for 1 h. Washed cells were stained with 1 μg of DAPI/ml in PBS containing 0.5% Triton X-100. Samples were run on an EPICS ELITE-ESP flow cytometer (Beckman Coulter), and data were analyzed with WinList, version 2.0, software (Verity Software House, Inc.).

Migration assay. The ability of endothelial cells to migrate toward FGF-2 or VEGF was measured by a Transwell filter assay (Corning Costar), as previously described (13). Briefly, polycarbonate filters (8.0-μm pores) of the upper chamber were coated with 50 μl of fibrinogen (2.5 mg/ml) or collagen type I (1 mg/ml) in PBS and allowed to dry overnight. Confluent plates of HMEC lines were trypsinized, washed twice with 10 μg of soybean trypsin inhibitor/ml, and resuspended in serum-free MCDB medium. HMEC (3.5×10^4) were placed in the upper chamber, and MCDB medium supplemented with FGF-2 (15 ng/ml) or VEGF (15 ng/ml) was placed in the lower chamber. As a control, MCDB medium without an added chemotactic factor was placed in the lower chamber. Following 16 h of incubation at 37°C, filters were washed in PBS, fixed in 4% paraformaldehyde, and stained with 0.5% crystal violet. After adherent cells were removed from the upper side of the filter with a cotton swab, cells that had migrated and adhered to the underside of the filter were counted with an inverted microscope.

Adhesion assay. High-binding 96-well plates (Corning Costar) were coated with 100 μl of the following matrix proteins/well at 20 μg/ml: fibrinogen, fibronectin, collagen type I, collagen type IV, and vitronectin. Control wells were coated with poly-L-lysine at 20 μg/ml. After incubation for 1 h at 37°C, all wells were aspirated and blocked with 4% bovine serum albumin in PBS for 30 min at room temperature, followed by washing with PBS. Single-cell suspensions were prepared by washing confluent cells once with PBS-based enzyme-free cell dissociation buffer (Gibco) and incubating the cells in the same buffer for 20 min at 37°C, as described previously (70). Following resuspension in a mixture of PBS-DMEM (4:1 [vol/vol]), 100 μl of the cell suspension at 6×10^5 cells/ml was added to each well and incubated at 37°C for 20 min. Plates were then gently washed with PBS, fixed in 4% paraformaldehyde, and stained with 0.5% crystal violet. Following solubilization of dye in 1% sodium dodecyl sulfate in PBS, absorbance was quantitated in an enzyme-linked immunosorbent assay plate reader at 570 nm, with background absorbance subtracted at 630 nm.

For adhesion modulation studies, HMEC lines were incubated with antibodies on ice for 20 min. Mouse IgG2a (Sigma) was used at 1:500 dilution; mouse monoclonal function-blocking anti-human β1-integrin antibody P4C10 was used at 1:100, 1:250, and 1:500 dilutions (Gibco) or at 2.5 μg/ml (Sigma); mouse monoclonal function-blocking anti-human αvβ3-integrin antibody LM609 (Chemicon) was used at 10 μg/ml; and mouse monoclonal function-activating anti-human β1-integrin antibody 8A2 was used at 1 μg/ml. For cells treated with both LM609 and P4C10, a concentration of 10 μg/ml and a 1:100 dilution, respectively, were used. For adhesion studies using P4C10, cells were seeded into wells coated with collagen type I and/or collagen type IV. For adhesion studies using 8A2, cells were seeded into wells coated with collagen type I.

Flow cytometry for integrin expression levels. HMEC lines were detached by incubation in PBS-based enzyme-free cell dissociation buffer for 20 min at 37°C. To reduce nonspecific binding, cells were incubated in PBS–10% heat-inactivated iron-supplemented calf serum for 30 min at 37°C. Primary antibodies LM609 (10 μg/ml; Chemicon), B44 (10 μg/ml; gift from J. A. Wilkins, University of Manitoba), and K20 (10 μg/ml; AMAC, Inc.) were added to the cells, and the cells and antibodies were allowed to incubate at 37°C for 30 min. A mouse IgG2a antibody (10 μg/ml; Sigma) was used as a control. Cells were washed twice in cold PBS, and a secondary antibody (goat anti-mouse IgG-FITC, 1:64 dilution; Sigma) was added, and the cells and the antibody were allowed to incubate for an additional 60 min in the dark on ice. After cells were washed twice in cold PBS, they were fixed in 4% paraformaldehyde. Samples were run on an EPICS ELITE-ESP flow cytometer (Beckman Coulter), and data were analyzed with WinList, version 2.0 (Verity Software House, Inc.).

Ligand-binding assay. The binding of soluble collagen type I to HMEC-LNCX and HMEC-Notch4IC lines was examined. HMEC lines were detached by incubation in PBS-based enzyme-free cell dissociation buffer for 20 min at 37°C. Cells (5×10^5) were incubated with FITC-conjugated collagen type I (16.9 molecules of FITC per molecule of collagen; Molecular Probes) in a volume of 100 μl at 0, 0.1, 1, 10, 100, 500, and 1,000 μg/ml. After 10 min of binding at 37°C, cells were washed three times in 1 ml of PBS and fixed in 4% paraformaldehyde. Samples were run on an EPICS ELITE-ESP flow cytometer (Beckman Coulter), and data were analyzed with WinList, version 2.0 (Verity Software House, Inc.). To determine the number of molecules of FITC-collagen type I bound per endothelial cell, a standard fluorescence curve was constructed by using Quantum 24 premixed microbeads (Bangs Laboratories, Inc.). Taking the molecular mass of FITC and collagen type I to be 390 Da and 300 kDa, respectively, and given an FITC/collagen ratio of 16.9, a curve of bound collagen (molecules) versus concentration (nanomolar) of FITC-collagen type I conjugate was generated.

RESULTS

Constitutively active Notch4 inhibits endothelial sprouting in vitro. Given the role of Notch in modulating cell fate decisions, we postulated that the endothelium-specific Notch4 would modulate endothelial sprouting. To address this issue, we generated HMEC lines that express a truncated intracellular form of human Notch4, Notch4IC, which is constitutively active. A previously described endothelial-sprouting assay which mimics the formation of capillary-like tubes in fibrin gels in vitro was used to evaluate the role of Notch4 in angiogenesis (40, 53). Using this in vitro assay, we found that activated Notch4 blocked spontaneous endothelial sprout formation on gelatin-coated beads, as well as sprouting in response to FGF-2 and VEGF (Fig. 1A and B). Moreover, the sprouts that formed from Notch4IC-expressing cell lines were noted to be shorter than those derived from cells transduced with the empty vector. Figure 1C shows the expression of Notch4IC protein in HMEC-Notch4IC as determined by immunoblotting. Expression of the Notch4IC construct in HMEC was also analyzed by immunofluorescence. We typically achieve transduction efficiencies between 50 and 80%. As expected with polyclonal cell lines, HMEC-Notch4IC display heterogeneity in staining for the Notch4IC protein (Fig. 1D). The majority of the Notch4IC protein localizes to the nuclei of HMEC-Notch4IC, which is typical of constitutively active Notch proteins (24).

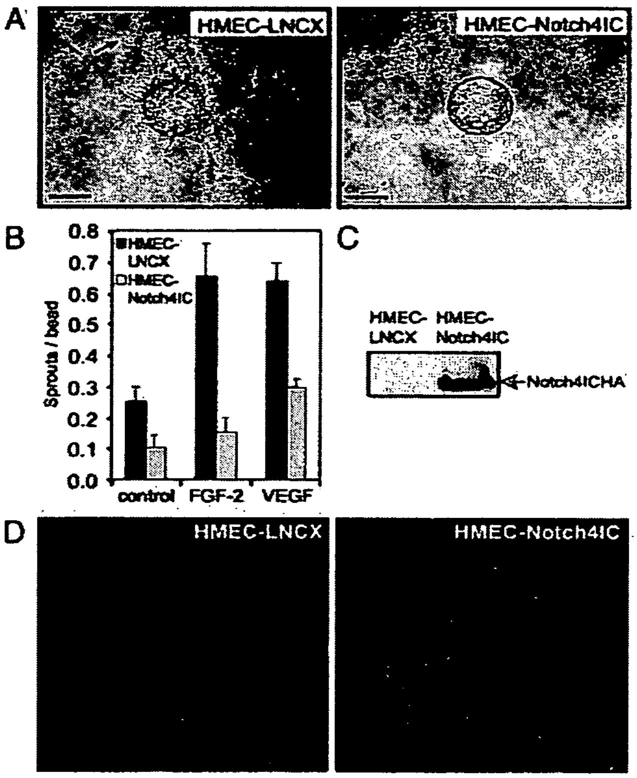


FIG. 1. Notch4 inhibits endothelial sprouting from gelatin-coated microcarrier beads in vitro. (A) Gelatin-coated microcarrier beads were seeded with HMEC-LNCX or HMEC-Notch4IC. When cells reached confluence on the beads, equal numbers of beads were embedded in fibrin gels supplemented with either FGF-2 (15 ng/ml) or VEGF (15 ng/ml). Bars, 100 μm. Arrows, endothelial sprouts of sufficient length to be counted. (B) Endothelial sprout formation quantitated after 3 days of incubation by counting the number of tube-like structures per microcarrier bead (sprouts per bead). Data are the means ± standard deviations from a single experiment done in triplicate and are representative of at least three independent experiments. (C) Expression of HA-tagged Notch4IC in HMEC lines by immunoblotting total cellular extracts with the anti-HA monoclonal antibody. (D) Immunofluorescence of HMEC-LNCX and HMEC-Notch4IC stained with Hoechst 33258, as well as an anti-HA primary antibody and a Texas red-conjugated secondary antibody to detect HA-tagged Notch4IC protein. Original magnification, ×40.

Because HMEC are a transformed endothelial cell line, we repeated the endothelial-sprouting assay using primary human umbilical vein endothelial cells (HUVEC) transduced with the Notch4IC construct or the empty vector. As for HMEC, activation of Notch4 in HUVEC blocked endothelial sprouting (data not shown).

Constitutively active Notch4 inhibits angiogenesis in vivo. To determine whether activation of Notch4 would inhibit angiogenesis in vivo, we used a chick CAM assay. The CAM functions as a respiratory structure for gas-nutrient exchange and undergoes intense vascularization (11), thus providing an excellent microenvironment for assessing angiogenesis. As previously described (9, 36), we generated avian retroviral packaging cell lines (Q2bn) transfected with the empty CK vector or CK-Notch4IC. On embryonic day 8, these CK producer lines, in the presence of VEGF, were placed on meshes on the chick CAM surface and incubated for an additional 4 days. The cells distribute throughout the mesh and secrete control virus or virus containing Notch4IC, which infects the surrounding proliferating cells, the majority of which are endothelial. CAMs transduced with the empty vector demonstrated normal angiogenesis in response to VEGF, whereas angiogenesis was markedly inhibited by the expression of Notch4IC (Fig. 2A and B). Expression of the Notch4IC protein in transfected Q2bn cells is shown in Fig. 2C.

Histological analysis was performed on sections of harvested CAMs. For H&E-stained sections, areas of the CAMs proximal to the Q2bn-containing mesh were analyzed. H&E staining of CK vector-transduced CAMs revealed the presence of numerous blood vessels in the subchorionic mesenchyme (Fig. 3A). In contrast, CAMs transduced with CK-Notch4IC exhibited a marked reduction in blood vessels close to the mesh (Fig. 3B). Immunohistochemistry was also performed on CAM sections and areas proximal to the mesh examined. Staining for the endothelium-specific marker vWF (65) confirmed the presence of blood vessels in CK vector-transduced CAMs (Fig. 3C). Notch4IC-transduced CAMs, on the other hand, showed minimal staining for vWF (Fig. 3D), confirming inhibition of blood vessel formation.

To assess expression of the HA-tagged Notch4IC protein in endothelial cells, serial sections were stained with antibodies against HA and vWF. Because Notch4IC-transduced CAMs were nearly devoid of small vessels proximal to the mesh, colocalization of staining was examined in vessels distant from the mesh. As expected for CK vector-transduced CAMs, vessels distant from the mesh did not stain for HA (Fig. 3E) but did stain for vWF (Fig. 3G). Analysis of Notch4IC-transduced CAMs demonstrated that vessels distant from the mesh exhibited costaining for HA (Fig. 3F) and vWF (Fig. 3H). Our findings suggest that expression of Notch4IC in vessels that feed the area of the mesh inhibits VEGF-induced endothelial sprouting and angiogenesis. To elucidate a possible mechanism(s) by which activated Notch4 inhibits endothelial sprouting in vitro and angiogenesis in vivo, we investigated the effects of Notch4IC expression on endothelial cell functions related to the angiogenic process by using various in vitro assays.

Notch4 inhibition of endothelial sprouting in vitro cannot be explained by reduced endothelial cell proliferation. Endothelial cell proliferation enables newly formed sprouts to increase in length and extend into the surrounding matrix. To determine whether reduced proliferation was a possible reason for the decreased sprouting of Notch4IC-expressing endothelial cells, we performed neutral red proliferation assays. When plated on normal tissue culture substrata, Notch4IC-expressing cells and control cells exhibited similar proliferation rates over 72 h (the incubation time for the endothelial-sprouting assay) (Fig. 4A). In fact, proliferation rates for cells grown in serum-containing medium were the same as those for cells grown in medium supplemented with FGF-2 or VEGF. Proliferation on fibrinogen- and collagen-coated surfaces was also investigated and was found to be equivalent for Notch4IC-expressing cells and control cells (data not shown).

To confirm that Notch4IC does not affect HMEC cell cycle kinetics, we performed flow cytometry on HMEC lines pulse-

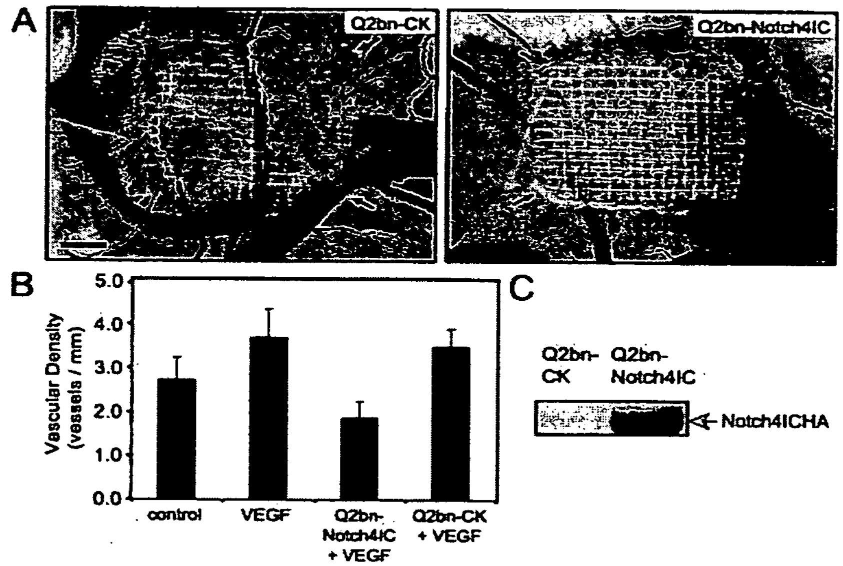


FIG. 2. Notch4 inhibits angiogenesis in the chick CAM in vivo. The avian retroviral packaging cell line Q2bn was transfected with empty vector CK (Q2bn-CK) or CK-Notch4IC (Q2bn-Notch4IC). On day 9, transfected Q2bn cell lines were placed onto nylon meshes on the CAM surface in the presence of VEGF (30 ng/ml). The grafted cells distribute throughout the mesh and secrete control virus or virus containing Notch4IC. Control CAMs were treated with medium alone or medium supplemented with VEGF. Images of the CAMs were captured on day 12. (A) CAMs treated with Q2bn-CK or Q2bn-Notch4IC cell lines in the presence of VEGF. Arrows, edges of the nylon mesh. Bars, 1 mm. (B) Vascular density quantitated after 4 days of incubation by counting the number of vessels that entered the mesh area and dividing by the perimeter of the mesh (vessels per millimeter). Data are the means ± standard errors from three experiments each done in replicates of four to six eggs. (C) Expression of HA-tagged Notch4IC in Q2bn cell lines verified by immunoblotting total cellular extracts with the anti-HA monoclonal antibody.

labeled with BrdU and costained with DAPI. Control and Notch4IC-expressing cells exhibited similar cell cycle distributions and levels of BrdU incorporation in the absence or presence of a growth factor (Fig. 4B shows representative samples of VEGF-stimulated HMEC-LNCX and HMEC-Notch4IC, and Fig. 4C shows distribution percentages). Overall, the proliferation studies performed demonstrate that control and Notch4IC-expressing cells proliferate at similar rates. Hence the inhibited sprouting of HMEC-Notch4IC in the in vitro endothelial-sprouting experiments cannot be explained by a decrease in proliferation of Notch4IC-expressing cells.

Notch4 inhibits endothelial cell migration through collagen but not fibrinogen. For capillaries to sprout, endothelial cells need to migrate toward a stimulus. To examine whether defective migration could explain the Notch4 inhibition of sprouting, we performed chemotaxis assays using Transwell filters coated with either fibrinogen or collagen. When filters were coated with fibrinogen, control cells and Notch4IC-expressing cells exhibited similar degrees of chemotaxis toward FGF-2 and VEGF (Fig. 5). Migration through collagen-coated filters toward FGF-2 or VEGF, however, for Notch4IC-expressing cells was less than that for control cells (Fig. 5). These data suggest that activated Notch4 does not affect the intrinsic motility of HMEC cells but influences endothelial cell migration in a matrix-dependent manner.

Notch4 promotes adhesion to extracellular matrix proteins through β1-integrins. Modulation of cell surface integrin levels as well as integrin affinity is a crucial event throughout the course of capillary tube formation (7, 28) and cell migration (46). Therefore, to explain the matrix-specific inhibition of HMEC-Notch4IC migration, we investigated whether Notch4 activation affects endothelial cell adhesion to extracellular matrix proteins. Figure 6A shows that Notch4IC-expressing cells exhibited increased adherence to various matrix proteins. In contrast, when adhesion was mediated by charge interactions alone, Notch4IC-expressing cells and control cells adhered to poly-L-lysine to the same degree. Regulation of αvβ3- and β1-integrins is required for angiogenesis (7, 21). Because activation of Notch4 promoted adhesion to the β1-integrin substrates tested (Fig. 6A), we postulated that the pattern of increased adhesion was due to effects of Notch4 activation on β1-integrin expression or function. Using function-blocking β1-integrin antibody P4C10 (12), we confirmed that the majority of the increased HMEC-Notch4IC adhesion to collagen type I (Fig. 6B and C) or collagen type IV (Fig. 6C) was mediated by β1-integrins. A function-blocking antibody directed against αvβ3-integrin (LM609) (14), however, did not affect HMEC-Notch4IC adhesion to collagen type I (Fig. 6B). LM609 concentrations of up to 20 μg/ml were tested, with no effect on collagen type I adhesion (data not shown). Interestingly, when LM609 and P4C10 were used in combination, the inhibition of HMEC-Notch4IC adhesion to collagen type I was less effective than when P4C10 was used alone (Fig. 6B). Although the reason(s) for the attenuated blocking is not clear, in part this may be due to steric hindrance.

We next tested whether Notch4IC affected the expression levels of αvβ3- and β1-integrins at the cell surface. Using flow cytometry, we demonstrated that neither αvβ3- nor β1-integrin levels were upregulated on the surface of HMEC-Notch4IC compared to levels for controls (Fig. 7A). In fact, in most

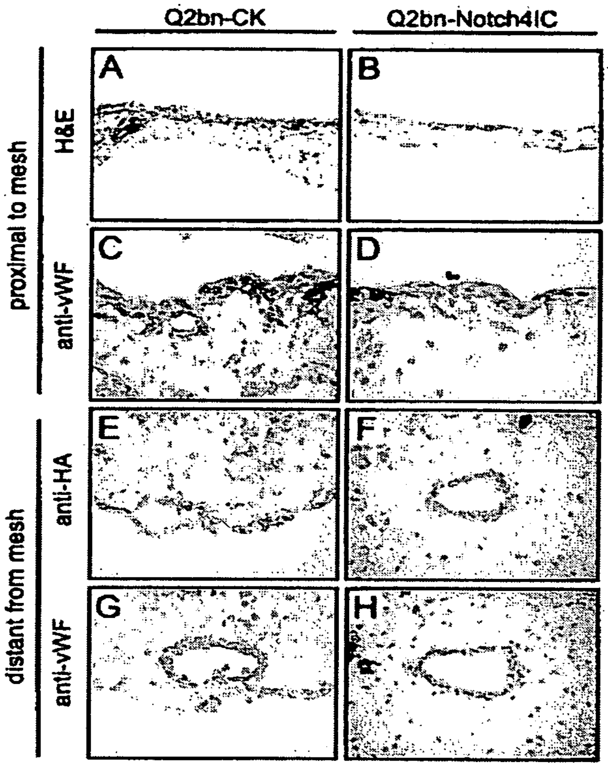
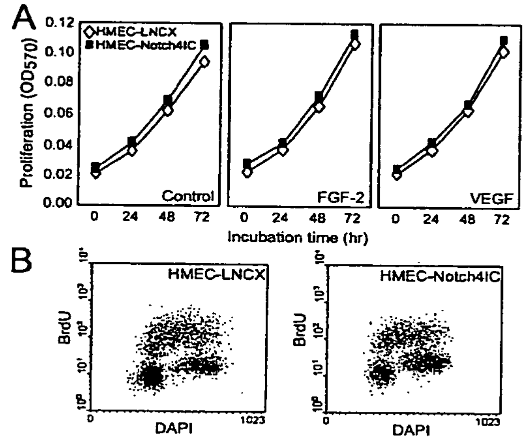


FIG. 3. Immunohistochemical analysis of Notch4IC expression in the CAM. Q2bn packaging cells transfected with the vector control (Q2bn-CK) or Notch4IC (Q2bn-Notch4IC) were placed onto nylon meshes on the CAM surface. Treated CAMs were harvested on day 12, and sections were prepared. (A to D) CAM sections proximal to mesh. Shown is H&E staining of Q2bn-CK-treated (A) and Q2bn-Notch4IC-treated (B) CAMs and anti-vWF staining of Q2bn-CK-treated (C) and Q2bn-Notch4IC-treated (D) CAMs. (E to H) CAM sections distant from mesh. Shown is anti-HA staining of Q2bn-CK-treated (E) and Q2bn-Notch4IC-treated (F) CAMs and anti-vWF staining of Q2bn-CK-treated (G) and Q2bn-Notch4IC-treated (H) CAMs. Original magnifications: ×40 (A to D, F, and H) and ×63 (E and G).

experiments, there was decreased β1-integrin, but not αvβ3-integrin, on the surface of HMEC-Notch4IC. Integrins can exist at the cell surface in at least two conformational states, a ligand-binding (active or high-affinity) conformation and a non-ligand-binding (inactive or low-affinity) conformation (33). Increased affinity of integrins for their ligand(s) can be regulated by intracellular events, a process referred to as inside-out signaling (33). Our findings of increased β1-integrin-mediated adhesion without increased β1-integrin expression suggest that Notch4IC may participate in an inside-out signaling process that promotes β1-integrin affinity. To test this hypothesis, we performed ligand-binding assays using FITC-conjugated collagen type I. The binding of soluble collagen type I to HMEC-Notch4IC was greater than that to control HMEC (Fig. 7B). Because HMEC-Notch4IC exhibit increased binding to soluble collagen type I (Fig. 7B) without a corresponding increase in total β1-integrin expression (Fig. 7A), our findings suggest that HMEC-Notch4IC display a greater number of



C

	HMEC-LNCX			HMEC-Notch4IC		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
Control	44 ± 1%	29 ± 4%	25 ± 3%	36 ± 4%	30 ± 4%	30 ± 7%
FGF-2	37 ± 4%	34 ± 2%	24 ± 1%	35 ± 3%	30 ± 5%	33 ± 3%
VEGF	41 ± 1%	33 ± 2%	26 ± 5%	38 ± 6%	32 ± 3%	27 ± 6%

FIG. 4. Notch4 does not inhibit HMEC proliferation. (A) Neutral red assay for proliferation. HMEC-LNCX and HMEC-Notch4IC proliferation in medium alone and in medium supplemented with FGF-2 (15 ng/ml) or VEGF (15 ng/ml) was assayed over 72 h. Data are the mean absorbances from a single experiment done in triplicate and are representative of at least three independent experiments. OD_{570}, optical density at 570 nm. (B) Cell cycle distribution for HMEC-LNCX and HMEC-Notch4IC stimulated with VEGF. Cells were stained with DAPI for total DNA content and pulse-labeled with BrdU to detect DNA synthesis and analyzed by flow cytometry. (C) Cell cycle distributions for HMEC-LNCX and HMEC-Notch4IC cultured in medium alone or medium supplemented with FGF-2 (15 ng/ml) or VEGF (15 ng/ml). Data are the means ± standard errors from three independent experiments.

β1-integrins in a high-affinity, active conformation, than control cells.

To confirm the increased proportion of active β1-integrin on the surface of HMEC-Notch4IC, Notch4IC-expressing cells and control cells were stained with antibodies that specifically recognize active β1-integrin (B44) (55, 78) or total β1-integrin

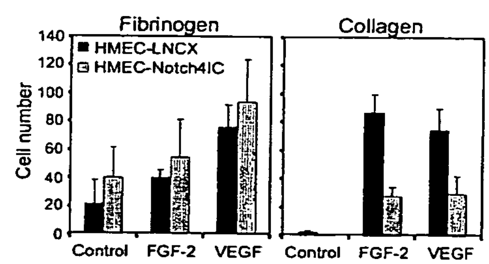


FIG. 5. Notch4 inhibits endothelial cell migration through collagen but not fibrinogen. Migration of HMEC-LNCX and HMEC-Notch4IC toward control medium and medium supplemented with FGF-2 (15 ng/ml) or VEGF (15 ng/ml) was assayed by using Transwell filters coated with fibrinogen or collagen type I. Following 16 h of incubation, cells that had migrated and adhered to the underside of the filter were stained and counted. Data are the means ± standard deviations from a single experiment done in triplicate and are representative of at least three independent experiments.

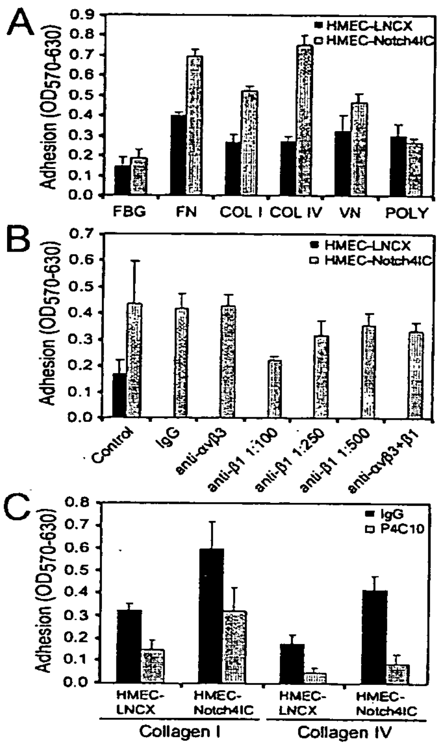


FIG. 6. Notch4 promotes endothelial cell adhesion to various extracellular matrix proteins through β1-integrins. (A) Adhesion of HMEC-LNCX and HMEC-Notch4IC to extracellular matrix proteins. Plates were coated with the following proteins: fibrinogen (FBG), fibronectin (FN), collagen type I (COL I), collagen type IV (COL IV), vitronectin (VN), and poly-L-lysine (POLY). Adherent cells were fixed, stained, and solubilized, and absorbance was read at 570 nm with background absorbance at 630 nm subtracted ($OD_{570-630}$). (B) Adhesion of HMEC-LNCX and HMEC-Notch4IC in the presence of function-blocking antibodies against αvβ3- and β1-integrins. Adhesion assays were performed on plates coated with collagen type I. HMEC-LNCX and HMEC-Notch4IC were preincubated with IgG2a (1:100 dilution), an anti-αvβ3 antibody (LM609; 10 μg/ml), and an anti-β1 antibody (P4C10; 1:100, 1:250, and 1:500 dilutions). For cells treated with both αvβ3 and β1 antibodies, 10 μg/ml and a 1:100 dilution, respectively, were used. (C) Adhesion of HMEC-LNCX and HMEC-Notch4IC in the presence of a function-blocking β1-integrin antibody. Adhesion assays were performed on plates coated with collagen type I or collagen type IV. Cells were preincubated with IgG2a (1:500 dilution) or an anti-β1 antibody (P4C10; 2.5 μg/ml). Adhesion data are the means ± standard deviations from a single experiment done in triplicate and are representative of at least three independent experiments.

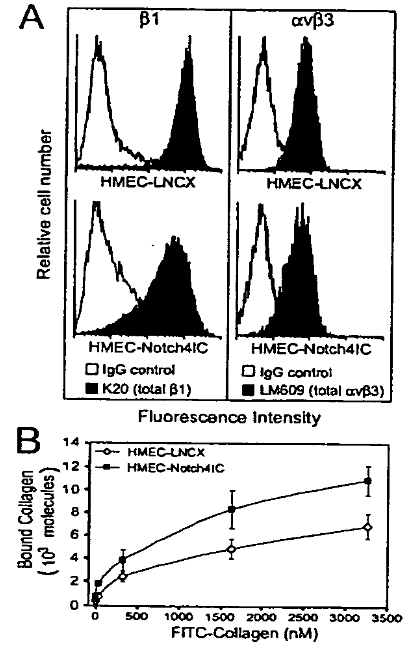


FIG. 7. Notch4 does not increase endothelial cell surface expression of β1-integrins but enhances binding of soluble collagen. (A) Surface expression of αvβ3- and β1-integrins on HMEC-LNCX and HMEC-Notch4IC. Cells were incubated with antibodies (IgG control, K20, and LM609) and analyzed by flow cytometry. Histograms are representative of at least three independent experiments. (B) Curves for binding of soluble collagen to HMEC-LNCX and HMEC-Notch4IC. FITC-conjugated collagen type I was incubated with cells at the indicated concentrations, and the samples were analyzed by flow cytometry. Binding data are the means ± standard deviations from two independent experiments.

(K20) (72) and mean fluorescence ratios (active β1/total β1) were determined by flow cytometry. Notch4IC-expressing cells, compared to control cells, displayed a greater proportion of β1-integrin receptors in a high-affinity state (Fig. 8A). Based on our findings, we reasoned that if β1-integrins expressed on HMEC-Notch4IC were already in a high-affinity state, we would not be able to further increase β1-integrin-mediated adhesion to collagen. Using a function-activating β1-integrin antibody (8A2) (42), we found that whereas HMEC-LNCX adhesion to collagen type I could be increased, 8A2 was unable to increase HMEC-Notch4IC adhesion to collagen type I (Fig. 8B). Taken together, our findings demonstrate that Notch4IC-expressing cells already display a fully active conformation of β1-integrins.

Increased β1-integrin-mediated adhesion plays a role in the Notch4 inhibition of endothelial sprouting. Our data suggest that the inhibited sprouting of Notch4IC-expressing cells in vitro may be explained in part by an increased affinity to gelatin-coated beads and that this high-affinity adhesive state (which presumably cannot be "turned off" due to the constitutive activation of Notch4) prevents the Notch4IC-expressing cells from migrating off the gelatin-coated beads and into the fibrin gel. This suggests that if Notch4IC-expressing cells were seeded onto beads by charge interaction rather than β1-integrin-mediated adhesion, the ability to form sprouts would be restored. To test this hypothesis, HMEC-Notch4IC were seeded onto dextran-coated microcarrier beads and the beads were embedded into fibrin gels. In this assay, we noted that HMEC-Notch4IC formed sprouts to a similar extent as HMEC-LNCX (Fig. 9). Hence in the absence of a β1-integrin substrate with which to interact, Notch4IC-expressing cells are capable of forming sprouts. As a control, dextran-coated beads were further coated with collagen type I and then seeded with HMEC-Notch4IC. Similar to results shown in Fig. 1B, sprout-

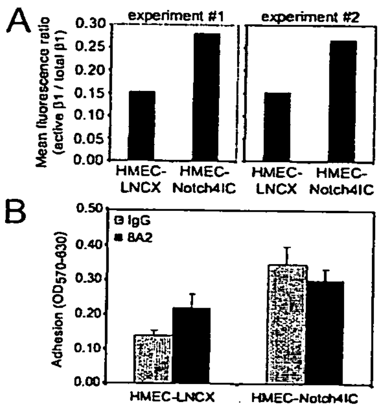


FIG. 8. Notch4-expressing cells display β1-integrins in a high-affinity conformation. (A) Mean fluorescence ratios of active β1 to total β1 on HMEC-LNCX and HMEC-Notch4IC. Cells were incubated with antibodies (B44, active β1; K20, total β1) and assessed by flow cytometry. Data are from two independent experiments. (B) HMEC-Notch4IC adhesion to collagen cannot be increased by function-activating β1-integrin antibodies. HMEC-LNCX and HMEC-Notch4IC preincubated with function-activating β1-integrin antibody 8A2 were added to collagen type I-coated wells. Adherent cells were fixed, stained, and solubilized, and absorbance was read at 570 nm with background absorbance at 630 nm subtracted ($OD_{570\text{-}630}$). Data are means ± standard deviations from a single experiment done in triplicate and are representative of at least three independent experiments. For the increased adhesion of HMEC-LNCX due to 8A2, the P value was 0.03 (analysis of variance).

ing of HMEC-Notch4IC from these collagen-recoated beads was inhibited (Fig. 9).

Activation of β1-integrins is sufficient to inhibit angiogenesis in vitro and in vivo. Our findings described thus far demonstrate that expression of activated Notch4 in endothelial cells inhibits angiogenesis both in vitro and in vivo, in part by promoting β1-integrin activation. To determine whether activation of β1-integrins alone (independent of constitutively active Notch4 expression) was sufficient to inhibit angiogenesis,

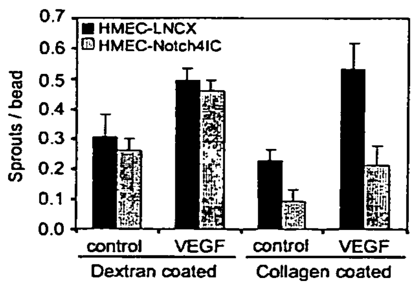


FIG. 9. Notch4 does not inhibit endothelial sprouting from dextran-coated microcarrier beads in vitro. Dextran-coated microcarrier beads were seeded with HMEC-LNCX or HMEC-Notch4IC. Equal numbers of beads were embedded in fibrin gels containing control medium or medium supplemented with VEGF (15 ng/ml). Endothelial sprout formation was quantitated after 3 days of incubation. As a control, dextran-coated beads were coated with collagen type I and were then seeded with HMEC lines. Data are the means ± standard deviations from a single experiment done in triplicate and are representative of at least three independent experiments.

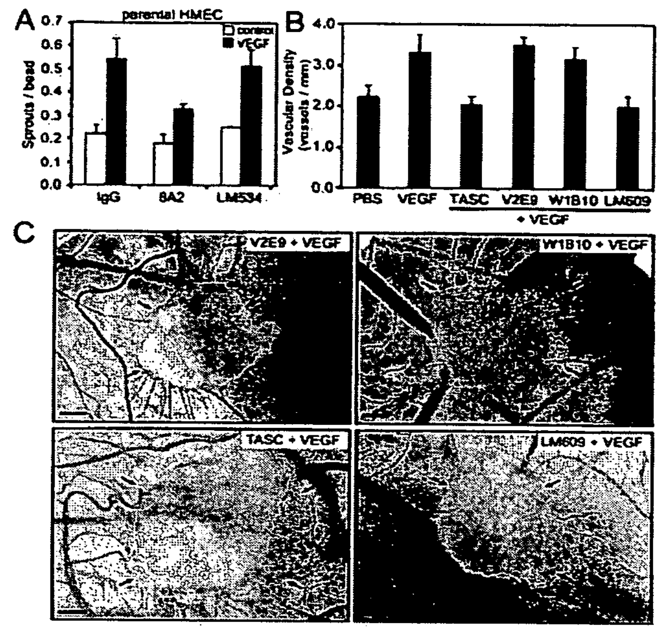


FIG. 10. Activation of β1-integrins alone, independent of Notch4 activation, is sufficient to inhibit endothelial sprouting in vitro and angiogenesis in vivo. (A) In vitro sprouting of parental HMEC from microcarrier beads coated with anti-β1-integrin antibodies. Dextran-coated microcarrier beads were preincubated with an IgG control antibody, a function-activating β1-integrin antibody (8A2), or a non-function-modifying β1-integrin antibody (LM534). Data are the means ± standard deviations from a single experiment done in triplicate and are representative of at least three independent experiments. (B) Angiogenesis in the chick CAM in the presence of anti-β1-integrin antibodies. The following antibodies were used: TASC (function-activating β1-integrin antibody), V2E9 (non-function-modifying β1-integrin antibody), W1B10 (function-blocking β1-integrin antibody), LM609 (function-blocking αvβ3-integrin antibody). Antibodies (10 μg/ml) plus VEGF (30 ng/ml) were loaded onto gelatin sponges, and the sponges were placed on the CAMs of day 8 embryos. As controls, sponges containing PBS or VEGF (30 ng/ml) were placed on CAMs. Angiogenesis was quantitated on day 10. Data are the means ± standard errors from two experiments each done in replicates of three to five eggs. (C) CAMs treated with V2E9, W1B10, TASC, and LM609 antibodies, all in the presence of VEGF. CAMs are representative of two independent experiments. Arrows, corners of the sponges. Bars, 1 mm.

we performed in vitro and in vivo experiments using antibodies that specifically activate β1-integrins.

Using the in vitro sprouting model with untransduced parental HMEC, we investigated the effect of function-activating β1-integrin antibody 8A2 on VEGF-induced sprouting. Dextran-coated beads were preincubated with either 8A2 or LM534, a non-function-modifying β1 antibody (72). These beads were subsequently seeded with parental HMEC and incubated for 3 days to allow parental HMEC to produce and secrete their own matrix proteins (many of which are β1-integrin substrates) onto the bead surface (44). Figure 10A shows that, whereas VEGF was able to induce sprouting of parental HMEC from beads coated with LM534, sprouting from 8A2-coated beads was reduced. Hence β1-integrin activation was sufficient to inhibit VEGF-induced endothelial

sprouting in vitro. The in vivo chick CAM assay was used to examine VEGF-induced angiogenesis in the presence of various anti-avian β1-integrin antibodies (Fig. 10B and C). CAMs were treated with TASC (a function-activating β1-integrin antibody) (16, 54), V2E9 (a non-function-modifying β1-integrin antibody) (31), or W1B10 (a function-blocking β1-integrin antibody) (16). Whereas VEGF was able to induce angiogenesis in CAMs treated with V2E9 and W1B10, CAMs treated with TASC exhibited decreased angiogenesis (Fig. 10B and C). In fact, angiogenesis in TASC-treated CAMs was reduced to a level similar to that for CAMs treated with LM609 (Fig. 10B and C), a function-blocking αvβ3-integrin antibody previously shown to attenuate VEGF-induced angiogenesis in the CAM (23). Taken together, our findings demonstrate that activation of β1-integrins, and hence increased adhesion through β1-integrins, is sufficient to inhibit VEGF-induced angiogenesis in vitro and in vivo.

DISCUSSION

There is good evidence to indicate that members of the Notch family of transmembrane receptors play an important role in regulating cell fate decisions and differentiation (2). More recently, several studies point to a role for Notch and its ligands in influencing vascular development. Mutations in Notch3 are responsible for the human vascular disorder cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, although in this case the defect appears to be mainly in vascular smooth muscle cells (37). Mutations in presenilin 1, a protein involved in Notch proteolytic processing, results in hemorrhaging (67, 79). Mice that are rendered null for Notch ligands Jagged1 and Delta1 exhibit vascular remodeling defects (80) and hemorrhaging (32), respectively. Antisense oligonucleotides directed against Jagged1 enhance FGF-2-induced endothelial tube formation in a collagen gel assay (81). Recently, a study examining the expression of four Notch receptors (Notch1 to -4) and five Notch ligands (Delta1, -3, and -4 and Jagged1 and -2) in the developing mouse vasculature was performed. Notch1, Notch3, Notch4, Delta4, Jagged1, and Jagged2 are all expressed in arteries but not veins (76). Notch2, Delta1, and Delta3, on the other hand, are not expressed in vessels (76). Nevertheless, a Notch2 hypomorphic allele disrupts vessel remodeling in multiple vascular beds (50). The combined loss of Notch4 and Notch1 functions due to gene targeting in the mouse results in defects in vascular remodeling (34, 45). Interestingly, expression of activated Notch4 in the mouse embryonic vasculature, under the control of the VEGF-R2 promoter, also results in vascular patterning defects (73). Although the last two studies demonstrate that both increases and decreases in Notch4 signaling result in a common vascular phenotype, disrupted blood vessel development, a mechanism(s) by which to explain this phenotype has not been elucidated.

The enforced expression of a constitutively active form of murine Notch4 in a mammary epithelial cell line has been shown to inhibit branching morphogenesis in a collagen gel assay (75). Because mammary epithelial tubulogenesis and blood vessel angiogenesis are similar morphogenic processes (52) and because Notch4 is primarily expressed in the endothelium (47, 74), we investigated whether enforced expression of activated Notch4 (Notch4IC) in endothelial cells could inhibit endothelial sprouting in vitro and angiogenesis in vivo. In an in vitro endothelial-tube formation assay, we show that Notch4IC inhibits spontaneous endothelial sprouting, as well as sprouting in response to FGF-2 and VEGF (Fig. 1). Furthermore, using an in vivo chick CAM assay, we demonstrate that Notch4IC expression is sufficient to inhibit VEGF-induced angiogenesis (Fig. 2 and 3).

Quiescent endothelial cells are normally anchored by their abluminal surface to a collagen-rich matrix (38). At the initiation of angiogenesis, the mature collagen-containing matrix is degraded and replaced by a provisional matrix of fibrin and fibronectin upon which endothelial cells migrate and proliferate (20, 59). The endothelial-sprouting assay used in our studies mimics angiogenesis in vivo. Specifically, microvascular endothelial cells are seeded as a monolayer onto gelatin-coated beads and are then induced by angiogenic factors to migrate into a fibrin matrix to form sprouts. We report that endothelial cells expressing Notch4IC exhibit inhibited sprouting in vitro (Fig. 1 and 9) and that this inhibition can be explained in part by an increase in HMEC-Notch4IC adhesion to collagen (Fig. 6 to 8). By enhancing cell adherence to collagen-coated beads, activated Notch4 prevents migration of the cells into the fibrin matrix. This is in accordance with our migration studies, where HMEC-Notch4IC migration through collagen, but not fibrinogen, was inhibited (Fig. 5). Proliferation rates, on the other hand, in HMEC-Notch4IC and control cells were found to be similar (Fig. 4). Our in vivo studies demonstrate that Notch4IC expression in the chick CAM inhibits VEGF-induced angiogenesis (Fig. 2 and 3). Based on our in vitro findings, the inhibition of angiogenesis in vivo may be due in part to enhanced endothelial cell adhesion to matrix proteins, thereby inhibiting vascular remodeling in the CAM.

Cell migration requires the coordinated activation and deactivation of integrins (46). As a cell migrates across a matrix, integrins at the leading edge of the cell adhere to the substrate (35). At the same time, receptors at the trailing edge of the cell detach from the substrate to allow the cell to progress forward (56). Thus, during the sprouting process of angiogenesis, integrin affinity states are constantly being modulated. The αvβ3-integrin has been shown to play a critical role in angiogenesis, but several studies also delineate the essential contribution of β1-integrins in endothelial morphogenesis (7, 21). Our data show that activated Notch4 increases endothelial cell adhesion (Fig. 6) and that enhanced β1-integrin affinity plays a role in this increased adhesion (Fig. 7 and 8).

Our work demonstrates that constitutive Notch4 activation inhibits vascular remodeling. Importantly, our studies provide a possible mechanism with which to explain the common vascular defects observed in mutant mice with either increased (73) or decreased (45) Notch signaling. Because Notch plays a role in cell fate decisions, Notch signaling must be precisely regulated and hence requires cessation of receptor signaling at certain times (2, 51, 77). Similarly, because cell adhesion influences cell functions such as migration and cell phenotype, modulation of cell adhesion must be strictly regulated (7, 26, 46, 57, 63). Therefore, it is possible that knocking out Notch4 and Notch1 results in a loss of cell-to-extracellular matrix adhesion and hence inhibited vascular remodeling, whereas constitutive Notch4 activation results in excessive cell-to-extracel-

lular matrix adhesion, thereby effectively fixing the cells in place. Taken together, our studies as well as the studies of Krebs et al. (45) and Uyttendaele et al. (73) reveal that altered Notch4 signaling results in disrupted blood vessel development.

Notch-like extracellular matrix protein Del1 has been shown to induce integrin signaling and angiogenesis by binding endothelial $\alpha v\beta 3$ and promoting migration (58). This is a case of signaling from the outside to the interior of the cell, as seen with many transmembrane receptors. In contrast, our studies suggest that activation of Notch4 propagates signals that induce an active, high-affinity conformation of the $\beta 1$-integrin. To our knowledge, this is the first report demonstrating that any Notch member can regulate inside-out signaling of integrins. We are currently in the process of examining the potential pathways contributing to modulation of $\beta 1$-integrin affinity by Notch4.

There is much evidence demonstrating that suppression of integrin activation is a physiological mechanism with which to control integrin-dependent cell adhesion and migration (33). In addition, regulation of integrin activation has been reported to precede differentiation in several cell types. Regulation of $\beta 1$-integrin activity in neurogenic and myogenic differentiation, two processes that are also modulated by Notch, has been reported (8, 54). In a baboon model, it has previously been shown that in uninjured saphenous arteries endothelial cells and vascular smooth muscle cells express an epitope characteristic of $\beta 1$-integrins in a high-affinity state (43). However, 6 weeks following balloon injury, regenerating endothelial cells did not express this ligand-induced epitope, although there was no decrease in the expression of total $\beta 1$-integrin (43). In the same study, activation of $\beta 1$-integrin with function-activating $\beta 1$ antibody 8A2 inhibited the migration of endothelial cells in vitro (43). Together, these findings suggest that activated $\beta 1$-integrin is required to maintain endothelial cells in a quiescent state, but, to repair arteries and possibly to allow neovascularization, dyshesion by downregulating $\beta 1$-integrin affinity is required. In fact, activation of $\beta 1$-integrins on human endothelial cells has been shown to inhibit capillary tube formation in collagen gels in vitro (25). We report that activation of $\beta 1$-integrins on endothelial cells, independent of Notch4 activation, inhibits endothelial sprouting in vitro (Fig. 10A). Furthermore, we demonstrate that $\beta 1$-integrin activation can inhibit angiogenesis in the chick CAM in vivo (Fig. 10B and C). In a previous study using function-blocking antibodies directed against specific α-integrin subunits, a combination of $\alpha 1$-blocking and $\alpha 2$-blocking antibodies was shown to inhibit VEGF-induced angiogenesis in a mouse Matrigel plug assay (66). These findings suggest that blocking $\alpha 1\beta 1$- and $\alpha 2\beta 1$-integrin function can inhibit VEGF-induced angiogenesis (66). Although these results may seem contradictory to our data demonstrating that blocking $\beta 1$-integrin function does not inhibit VEGF-induced angiogenesis in the chick CAM (Fig. 10B and C), it is important to note that the effect of function-blocking and -activating antibodies directed against the $\beta 1$-integrin subunit in the Matrigel plug assay was not reported. Because numerous $\alpha\beta 1$-integrin heterodimers are implicated in angiogenesis (4, 17), blocking the function of only the $\alpha 1$ and $\alpha 2$ subunits may result in a different phenotype from that seen when the function of all $\beta 1$-integrins is blocked. Alternatively, the different results may reflect intrinsic differences in the experimental models used. Indeed, function-blocking $\beta 1$-integrin antibody CSAT has been reported to disrupt vascular development and lumen formation when microinjected into quail embryos (18), whereas the same CSAT antibody does not affect FGF-2- or tumor necrosis factor alpha-induced angiogenesis in the chick CAM (10).

Because Notch4 expression is restricted to the endothelium (74) and because Notch4 is the only Notch receptor expressed in the capillary endothelium (76), our findings implicate selective activation of Notch4 as a possible method by which to inhibit angiogenesis in pathological contexts. However, because our studies involve a constitutively active, overexpressed form of Notch4 in endothelial cells, the physiological relevance of the data must be interpreted with caution. Further studies using ligands specific for Notch4 will be important to determine whether modulated activation of Notch4 also inhibits angiogenesis. Recent studies suggest that Delta-like4 (Dll4) may be a potential ligand for Notch4, based on similar expression patterns for the two proteins (45, 69). However, it remains to be seen whether Dll4 can physically interact with and activate Notch4 and induce Notch4 signaling.

Hence the ability of Notch4 to inhibit endothelial sprouting in vitro and angiogenesis in vivo may be related in part to its ability to increase the ligand-binding affinity of $\beta 1$-integrins, as we have demonstrated in this report. Other potential mechanisms, however, may act in concert with $\beta 1$-integrin activation to mediate the observed Notch4 effect.

ACKNOWLEDGMENTS

We thank Mina Bissell and Nancy Boudreau for providing the avian retroviral vector CK and Nancy Boudreau for advice on the chick CAM assay. We also thank Kelly McNagny for the Q2bn cell line, John Harlan for the 8A2 antibody, John A. Wilkins for the B44 antibody, and Louis F. Reichardt for the TASC antibody. The V2E9 antibody, developed by Alan F. Horwitz, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, Iowa. Thanks are also due to Penny Costello for assistance with the setup of the chick CAM assay and Linda Hughes for immunohistochemical staining of CAM sections.

This research was supported by grants to A.K. from the Heart and Stroke Foundation of British Columbia and the Yukon and the National Cancer Institute of Canada with funds from the Canadian Cancer Society and the Canadian Breast Cancer Foundation (BC Chapter) and to L.L. from the Stowers Institute for Medical Research. B.L. was supported by a Doctoral Research Award from the Heart and Stroke Foundation of Canada. K.G.L. was supported by a Doctoral Research Award from the Canadian Institutes of Health Research and a Predoctoral Fellowship Award from the Department of the Army (DAMD17-01-1-0164). The U.S. Army Medical Research Acquisition Activity, Fort Detrick, Md., is the awarding and administering acquisition office. A.K. is a Clinician-Scientist of the Canadian Institutes of Health Research and a Scholar of the Michael Smith Foundation for Health Research.

REFERENCES

1. Ades, E. W., F. J. Candal, R. A. Swerlick, V. G. George, S. Summers, D. C. Bosse, and T. J. Lawley. 1992. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. J. Investig. Dermatol. 99:683–690.
2. Artavanis-Tsakonas, S., M. D. Rand, and R. J. Lake. 1999. Notch signaling: cell fate control and signal integration in development. Science 284:770–776.
3. Auerbach, W., and R. Auerbach. 1994. Angiogenesis inhibition. Pharmacol. Ther. 63:265–311.
4. Bauer, J., M. Margolis, C. Schreiner, C. J. Edgell, J. Azizkhan, E. Lazarowski, and R. L. Juliano. 1992. In vitro model of angiogenesis using a human endothelium-derived permanent cell line: contributions of induced gene expression, G-proteins, and integrins. J. Cell Physiol. 153:437–449.

5. Bazoni, G., E. Dejana, and M. G. Lampugnani. 1999. Endothelial adhesion molecules in the development of the vascular tree: the garden of forking paths. Curr. Opin. Cell Biol. 11:573–581.
6. Bigas, A., D. I. K. Martin, and L. A. Milner. 1998. Notch1 and Notch2 inhibit myeloid differentiation in response to different cytokines. Mol. Cell. Biol. 18:2324–2333.
7. Bloch, W., E. Forsberg, S. Lentini, C. Brakebusch, K. Martin, H. W. Krell, U. H. Weidle, K. Addicks, and R. Fassler. 1997. β1 integrin is essential for teratoma growth and angiogenesis. J. Cell Biol. 139:265–278.
8. Boettiger, D., M. Enomoto-Iwamoto, H. Y. Yoon, U. Hofer, A. S. Menko, and R. Chiquet-Ehrismann. 1995. Regulation of integrin alpha 5 beta 1 affinity during myogenic differentiation. Dev. Biol. 169:261–272.
9. Boudreau, N., C. Andrews, A. Srebrow, A. Ravanpay, and D. A. Cheresh. 1997. Induction of the angiogenic phenotype by Hox D3. J. Cell Biol. 139:257–264.
10. Brooks, P. C., R. A. Clark, and D. A. Cheresh. 1994. Requirement of vascular integrin alpha v beta 3 for angiogenesis. Science 264:569–571.
11. Brooks, P. C., A. M. Montgomery, and D. A. Cheresh. 1999. Use of the 10-day-old chick embryo model for studying angiogenesis. Methods Mol. Biol. 129:257–269.
12. Carter, W. G., E. A. Wayner, T. S. Bouchard, and P. Kaur. 1990. The role of integrins alpha 2 beta 1 and alpha 3 beta 1 in cell-cell and cell-substrate adhesion of human epidermal cells. J. Cell Biol. 110:1387–1404.
13. Celis, J. E. E. 1998. Cell biology: a laboratory handbook, 2nd ed. Academic Press, San Diego, Calif.
14. Cheresh, D. A. 1987. Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor. Proc. Natl. Acad. Sci. USA 84:6471–6475.
15. Cockerill, G. W., J. R. Gamble, and M. A. Vadas. 1995. Angiogenesis: models and modulators. Int. Rev. Cytol. 159:113–160.
16. Cruz, M. T., C. L. Dalgard, and M. J. Ignatius. 1997. Functional partitioning of beta1 integrins revealed by activating and inhibitory mAbs. J. Cell Sci. 110:2647–2659.
17. Davis, C. M., S. C. Danehower, A. Laurenza, and J. L. Molony. 1993. Identification of a role of the vitronectin receptor and protein kinase C in the induction of endothelial cell vascular formation. J. Cell. Biochem. 51:206–218.
18. Drake, C. J., L. A. Davis, and C. D. Little. 1992. Antibodies to beta 1-integrins cause alterations of aortic vasculogenesis, in vivo. Dev. Dyn. 193:83–91.
19. Duriez, P. J., F. Wong, K. Dorovini-Zis, R. Shahidi, and A. Karsan. 2000. A1 functions at the mitochondria to delay endothelial apoptosis in response to tumor necrosis factor. J. Biol. Chem. 275:18099–18107.
20. Dvorak, H. F., L. F. Brown, M. Detmar, and A. M. Dvorak. 1995. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am. J. Pathol. 146:1029–1039.
21. Eliceiri, B. P., and D. A. Cheresh. 1999. The role of alphav integrins during angiogenesis: insights into potential mechanisms of action and clinical development. J. Clin. Investig. 103:1227–1230.
22. Ellison, L. W., J. Bird, D. C. West, A. L. Soreng, T. C. Reynolds, S. D. Smith, and J. Sklar. 1991. TAN-1, the human homologue of the Drosophila *Notch* gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell 66:649–661.
23. Friedlander, M., P. C. Brooks, R. W. Shaffer, C. M. Kincaid, J. A. Varner, and D. A. Cheresh. 1995. Definition of two angiogenic pathways by distinct alpha v integrins. Science 270:1500–1502.
24. Furriols, M., and S. Bray. 2000. Dissecting the mechanisms of suppressor of hairless function. Dev. Biol. 227:520–532.
25. Gamble, J., G. Meyer, L. Noack, J. Furze, L. Matthias, N. Kovach, J. Harlan, and M. Vadas. 1999. Beta(1) integrin activation inhibits in vitro tube formation: effects on cell migration, vacuole coalescence and lumen formation. Endothelium 7:23–34.
26. Gamble, J. R., L. J. Matthias, G. Meyer, P. Kaur, G. Russ, R. Faull, M. C. Berndt, and M. A. Vadas. 1993. Regulation of in vitro capillary tube formation by anti-integrin antibodies. J. Cell Biol. 121:931–943.
27. Garces, C., M. J. Ruizhidalgo, J. F. Demora, C. Park, L. Miele, J. Goldstein, E. Bonvini, A. Porras, and J. Laborda. 1997. Notch-1 controls the expression of fatty acid-activated transcription factors and is required for adipogenesis. J. Biol. Chem. 272:29729–29734.
28. Grant, D. S., K. I. Tashiro, B. Segui-Real, Y. Yamada, G. R. Martin, and H. K. Kleinman. 1989. Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures *in vitro*. Cell 58:933–943.
29. Greenwald, I. 1994. Structure/function studies of lin-12/Notch proteins. Curr. Biol. 4:556–562.
30. Hanahan, D., and J. Folkman. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86:353–364.
31. Hayashi, Y., B. Haimovich, A. Reszka, D. Boettiger, and A. Horwitz. 1990. Expression and function of chicken integrin beta 1 subunit and its cytoplasmic domain mutants in mouse NIH 3T3 cells. J. Cell Biol. 110:175–184.
32. Hrabe de Angelis, M., J. McIntyre II, and A. Gossler. 1997. Maintenance of somite borders in mice requires the Delta homologue Dll1. Nature 386:717–721.
33. Hughes, P. E., and M. Pfaff. 1998. Integrin affinity modulation. Trends Cell Biol. 8:359–364.
34. Huppert, S. S., A. Le, E. H. Schroeter, J. S. Mumm, M. T. Saxena, L. A. Milner, and R. Kopan. 2000. Embryonic lethality in mice homozygous for a processing-deficient allele of Notch1. Nature 405:966–970.
35. Huttenlocher, A., M. H. Ginsberg, and A. F. Horwitz. 1996. Modulation of cell migration by integrin-mediated cytoskeletal linkages and ligand-binding affinity. J. Cell Biol. 134:1551–1562.
36. Jiang, B. H., J. Z. Zheng, M. Aoki, and P. K. Vogt. 2000. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. Proc. Natl. Acad. Sci. USA 97:1749–1753.
37. Joutel, A., C. Corpechot, A. Ducros, K. Vahedi, H. Chabriat, P. Mouton, S. Alamowitch, V. Domenga, M. Cecillion, E. Marechal, J. Maciazek, C. Vayssiere, C. Cruaud, E. A. Cabanis, M. M. Ruchoux, J. Weissenbach, J. F. Bach, M. G. Bousser, and E. Tournier-Lasserve. 1996. Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. Nature 383:707–710.
38. Karsan, A., and J. M. Harlan. 1999. The blood vessel wall, p. 1770–1782. *In* R. Hoffman, E. J. J. Benz, S. J. Shattil, B. Furie, H. J. Cohen, L. E. Silberstein, and P. McGlave (ed.), Hematology: basic principles and practice. Churchill Livingstone, New York, N.Y.
39. Karsan, A., E. Yee, and J. M. Harlan. 1996. Endothelial cell death induced by tumor necrosis factor α is inhibited by the Bcl-2 family member, A1. J. Biol. Chem. 271:27201–27204.
40. Koblizek, T. I., C. Weiss, G. D. Yancopoulos, U. Deutsch, and W. Risau. 1998. Angiopoietin-1 induces sprouting angiogenesis *in vitro*. Curr. Biol. 8:529–532.
41. Kopan, R., J. S. Nye, and H. Weintraub. 1994. The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of *MyoD*. Development 120:2385–2396.
42. Kovach, N. L., T. M. Carlos, E. Yee, and J. M. Harlan. 1992. A monoclonal antibody to beta 1 integrin (CD29) stimulates VLA-dependent adherence of leukocytes to human umbilical vein endothelial cells and matrix components. J. Cell Biol. 116:499–509.
43. Koyama, N., J. Seki, S. Vergel, E. J. Mattsson, T. Yednock, N. L. Kovach, J. M. Harlan, and A. W. Clowes. 1996. Regulation and function of an activation-dependent epitope of the beta 1 integrins in vascular cells after balloon injury in baboon arteries and in vitro. Am. J. Pathol. 148:749–761.
44. Kramer, R. H., G. M. Fuh, and M. A. Karasek. 1985. Type IV collagen synthesis by cultured human microvascular endothelial cells and its deposition into the subendothelial basement membrane. Biochemistry 24:7423–7430.
45. Krebs, L. T., Y. Xue, C. R. Norton, J. R. Shutter, M. Maguire, J. P. Sundberg, D. Gallahan, V. Closson, J. Kitajewski, R. Callahan, G. H. Smith, K. L. Stark, and T. Gridley. 2000. Notch signaling is essential for vascular morphogenesis in mice. Genes Dev. 14:1343–1352.
46. Lauffenburger, D. A., and A. F. Horwitz. 1996. Cell migration: a physically integrated molecular process. Cell 84:359–369.
47. Li, L., G. M. Huang, A. B. Banta, Y. Deng, T. Smith, P. Dong, C. Friedman, L. Chen, B. J. Trask, T. Spies, L. Rowen, and L. Hood. 1998. Cloning, characterization, and the complete 56.8-kilobase DNA sequence of the human Notch4 gene. Genomics 51:45–58.
48. Li, L., I. D. Krantz, Y. Deng, A. Genin, A. B. Banta, C. C. Collins, M. Qi, B. J. Trask, L. Kuo, J. Cochran, T. Costa, M. E. M. Pierpont, E. B. Rand, D. A. Piccoli, L. Hood, and N. B. Spinner. 1997. Alagille syndrome is caused by mutations in human *Jagged1*, which encodes a ligand for Notch1. Nat. Genet. 16:243–251.
49. Lowik, C. W., M. J. Alblas, M. van de Ruit, S. E. Papapoulos, and G. van der Pluijm. 1993. Quantification of adherent and nonadherent cells cultured in 96-well plates using the supravital stain neutral red. Anal. Biochem. 213:426–433.
50. McCright, B., X. Gao, L. Shen, J. Lozier, Y. Lan, M. Maguire, D. Herzlinger, G. Weinmaster, R. Jiang, and T. Gridley. 2001. Defects in development of the kidney, heart and eye vasculature in mice homozygous for a hypomorphic Notch2 mutation. Development 128:491–502.
51. Milner, L. A., and A. Bigas. 1999. Notch as a mediator of cell fate determination in hematopoiesis: evidence and speculation. Blood 93:2431–2448.
52. Montesano, R., J. V. Soriano, K. M. Malinda, M. L. Ponce, A. Bafico, H. K. Kleinman, D. P. Bottaro, and S. A. Aaronson. 1998. Differential effects of hepatocyte growth factor isoforms on epithelial and endothelial tubulogenesis. Cell Growth Differ. 9:355–365.
53. Nehls, V., and D. Drenckhahn. 1995. A novel, microcarrier-based *in vitro* assay for rapid and reliable quantification of three-dimensional cell migration and angiogenesis. Microvasc. Res. 50:311–322.
54. Neugebauer, K. M., and L. F. Reichardt. 1991. Cell-surface regulation of beta 1-integrin activity on developing retinal neurons. Nature 350:68–71.
55. Ni, H., A. Li, N. Simonsen, and J. A. Wilkins. 1998. Integrin activation by dithiothreitol or Mn2+ induces a ligand-occupied conformation and exposure of a novel NH2-terminal regulatory site on the beta1 integrin chain. J. Biol. Chem. 273:7981–7987.
56. Palecek, S. P., A. Huttenlocher, A. F. Horwitz, and D. A. Lauffenburger.

1998. Physical and biochemical regulation of integrin release during rear detachment of migrating cells. J. Cell Sci. 111:929–940.
57. Palecek, S. P., J. C. Loftus, M. H. Ginsberg, D. A. Lauffenburger, and A. F. Horwitz. 1997. Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. Nature 385:537–540.
58. Penta, K., J. A. Varner, L. Liaw, C. Hidai, R. Schatzman, and T. Quertermous. 1999. Del1 induces integrin signaling and angiogenesis by ligation of $\alpha v\beta 3$. J. Biol. Chem. 274:11101–11109.
59. Pepper, M. S. 1997. Manipulating angiogenesis: from basic science to the bedside. Arterioscler. Thromb. Vasc. Biol. 17:605–619.
60. Rak, J., and R. S. Kerbel. 1997. bFGF and tumor angiogenesis—back in the limelight? Nat. Med. 3:1083–1084.
61. Rebay, I., R. G. Fehon, and S. Artavanis-Tsakonas. 1993. Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. Cell 74:319–329.
62. Risau, W. 1997. Mechanisms of angiogenesis. Nature 386:671–674.
63. Ruoslahti, E., and E. Engvall. 1997. Integrins and vascular extracellular matrix assembly. J. Clin. Investig. 99:1149–1152.
64. Schroeter, E. H., J. A. Kisslinger, and R. Kopan. 1998. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature 393:382–386.
65. Sehested, M., and K. Hou-Jensen. 1981. Factor VIII related antigen as an endothelial cell marker in benign and malignant diseases. Virchows Arch. A Pathol. Anat. Histol. 391:217–225.
66. Senger, D. R., K. P. Claffey, J. E. Benes, C. A. Perruzzi, A. P. Sergiou, and M. Detmar. 1997. Angiogenesis promoted by vascular endothelial growth factor: regulation through alpha1beta1 and alpha2beta1 integrins. Proc. Natl. Acad. Sci. USA 94:13612–13617.
67. Shen, J., R. T. Bronson, D. F. Chen, W. Xia, D. J. Selkoe, and S. Tonegawa. 1997. Skeletal and CNS defects in Presenilin-1-deficient mice. Cell 89:629–639.
68. Shirayoshi, Y., Y. Yuasa, T. Suzuki, K. Sugaya, E. Kawase, T. Ikemura, and N. Nakatsuji. 1997. Proto-oncogene of int-3, a mouse Notch homologue, is expressed in endothelial cells during early embryogenesis. Genes Cells 2:213–224.
69. Shutter, J. R., S. Scully, W. Fan, W. G. Richards, J. Kitajewski, G. A. Deblandre, C. R. Kintner, and K. L. Stark. 2000. Dll4, a novel Notch ligand expressed in arterial endothelium. Genes Dev. 14:1313–1318.
70. Sriramarao, P., M. Mendler, and M. A. Bourdon. 1993. Endothelial cell attachment and spreading on human tenascin is mediated by alpha 2 beta 1 and alpha v beta 3 integrins. J. Cell Sci. 105:1001–1012.
71. Struhl, G., and A. Adachi. 1998. Nuclear access and action of *Notch* in vivo. Cell 93:649–660.
72. Takada, Y., and W. Puzon. 1993. Identification of a regulatory region of integrin beta 1 subunit using activating and inhibiting antibodies. J. Biol. Chem. 268:17597–17601.
73. Uyttendaele, H., J. Ho, J. Rossant, and J. Kitajewski. 2001. Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. Proc. Natl. Acad. Sci. USA 98:5643–5648.
74. Uyttendaele, H., G. Marazzi, G. Wu, Q. Yan, D. Sassoon, and J. Kitajewski. 1996. *Notch4/Int-3*, a mammary proto-oncogene, is an endothelial cell-specific mammalian *Notch* gene. Development 122:2251–2259.
75. Uyttendaele, H., V. J. Soriano, R. Montesano, and J. Kitajewski. 1998. Notch4 and Wnt-1 proteins function to regulate branching morphogenesis of mammary epithelial cells in an opposing fashion. Dev. Biol. 196:204–217.
76. Villa, N., L. Walker, C. E. Lindsell, J. Gasson, M. L. Iruela-Arispe, and G. Weinmaster. 2001. Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. Mech. Dev. 108:161–164.
77. Weinmaster, G. 2000. Notch signal transduction: a real rip and more. Curr. Opin. Genet. Dev. 10:363–369.
78. Wilkins, J. A., A. Li, H. Ni, D. G. Stupack, and C. Shen. 1996. Control of beta1 integrin function: Localization of stimulatory epitopes. J. Biol. Chem. 271:3046–3051.
79. Wong, P. C., H. Zheng, H. Chen, M. W. Becher, D. J. Sirinathsinghji, M. E. Trumbauer, H. Y. Chen, D. L. Price, L. H. van der Ploeg, and S. S. Sisodia. 1997. Presenilin 1 is required for Notch1 and Dll1 expression in the paraxial mesoderm. Nature 387:288–292.
80. Xue, Y., X. Gao, C. E. Lindsell, C. R. Norton, B. Chang, C. Hicks, M. Gendron-Maguire, E. B. Rand, G. Weinmaster, and T. Gridley. 1999. Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. Hum. Mol. Genet. 8:723–730.
81. Zimrin, A. B., M. S. Pepper, G. A. McMahon, F. Nguyen, R. Montesano, and T. Maciag. 1996. An antisense oligonucleotide to the notch ligand jagged enhances fibroblast growth factor-induced angiogenesis in vitro. J. Biol. Chem. 271:32499–32502.

Exhibit C

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [6] : A61K 48/00, 38/17, 38/18, C07K 14/435, 14/485, 16/28, C12N 15/11, C07H 21/04 — A1

(11) International Publication Number: WO 97/45143

(43) International Publication Date: 4 December 1997 (04.12.97)

(21) International Application Number: PCT/US97/09407

(22) International Filing Date: 30 May 1997 (30.05.97)

(30) Priority Data:
60/018,841 31 May 1996 (31.05.96) US

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(71) Applicants (*for all designated States except US*): THE NATIONAL AMERICAN RED CROSS [US/US]; 430 17th Street, N.W., Washington, DC 20006 (US). UNIVERSITE DE GENEVE [CH/CH]; 1, rue Michel Servet, CH-1211 Genève 4 (CH).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): ZIMRIN, Ann, B. [US/US]; 1235 Crows Foot Road, Marriottsville, MD 21104 (US). MACIAG, Thomas [US/US]; 6050 Valerian Lane, Rockville, MD 20852 (US). PEPPER, Michael, S. [ZA/CH]; 6, chemin-du-chateau, CH-1245 Collonge-Bellerive (CH). MONTESANO, Roberto [IT/CH]; 39, route de Frontenex, CH-1207 Genève (CH). WONG, Michael, K., K. [CA/US]; 16408 Keats Terrace, Derwood, MD 20855 (US).

(74) Agent: MCCONATHY, Evelyn, H.; Law Offices of Evelyn H. McConathy, 11723 Quay Road, Oakton, VA 22124 (US).

(54) Title: THERAPEUTIC AND DIAGNOSTIC METHODS AND COMPOSITIONS BASED ON JAGGED/NOTCH PROTEINS AND NUCLEIC ACIDS

(57) Abstract

This invention relates to therapeutic and diagnostic methods and compositions based on Jagged/Notch proteins and nucleic acids, and on the role of their signaling pathway in endothelial cell migration and/or differentiation. In addition, this invention provides a substantially purified Jagged protein, as well as a substantially purified nucleic acid molecule or segment thereof encoding Jagged protein, or a functionally equivalent derivative, or allelic or species variant thereof.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

THERAPEUTIC AND DIAGNOSTIC METHODS AND COMPOSITIONS BASED ON JAGGED/NOTCH PROTEINS AND NUCLEIC ACIDS

RELATED APPLICATIONS

This patent application was originally filed as U.S. Provisional Application 60/018,841 on May 30, 1996.

STATEMENT OF GOVERNMENT RIGHTS IN THE INVENTION

Part of the work performed during the development of this invention utilized U.S. Government funds and NIH grants. Thus, the U.S. Government may have certain rights in the invention.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to therapeutic and diagnostic methods and compositions based on Jagged/Notch proteins and nucleic acids, and on the role of their signaling pathway in endothelial cell migration and/or differentiation.

Background of the Invention

The functional integrity of the human vascular system is maintained by the endothelial cell which monitors the non-thrombogenic interface between blood and tissue *in vivo*. Thus, factors that influence human endothelial cell function may contribute significantly to the regulation and maintenance of homeostasis (*see*, Maciag, in Progress in Hemostasis and Thrombosis, T. Spaet, ed. (New York: A.R. Liss), pp.167-182 (1984); Folkman and Klagsburn, *Science 235*:442-447 (1987); Burgess and Maciag, *Annu. Rev. Biochem. 58*:575-606 (1989)). Likewise, events that perturb this complex equilibrium are relevant to the pathophysiology of human disease states in which cellular components of the vascular tree are active participants including, *e.g.*, atherogenesis, coronary insufficiency, hypertension, rheumatoid arthritis, solid tumor growth and metastasis, and wound repair.

Since the endothelium is present in all organs and tissues, endothelial cell function is also fundamental to the physiology and integration of these multicellular systems. This includes the ability to monitor and interface with repair systems that employ the tightly regulated

inflammatory, angiogenic and neurotropic responses. Indeed, biochemical signals that are responsible for the modification of these responses have been well characterized as polypeptide growth factors and cytokines; however, their mechanisms of operation have, prior to the present invention, been poorly understood, impeding their acceptance as valuable tools in clinical management.

A major accomplishment of modern biology has been the recognition that structural elements responsible for physiologic functions are conserved throughout the animal kingdom. Genetic analysis of yeast, *C. elegans*, *Xenopus*, Zebra fish, and *Drosophila*, among others, have provided new insight into the regulation of the cell cycle, organelle biosynthesis and trafficking, cell fate and lineage decisions during development, as well as providing the fundamental principles for transcriptional/translational/post-translational regulation. Indeed, the conservation of structure-function principles exhibited by such systems has generated new insight into these and other regulatory systems utilized by mammalian cells. Moreover, a resolution of the genetic structure of the mammalian homologs for such genes in non-mammalian species has often led to a discernment of their function in mammals, even though the delineation of the function of a particular, homologous mammalian gene or gene fragment may well be serendipitous. In many cases, it is the result produced by expression and differential cDNA cloning strategies that manifest mammalian DNA sequences with homology to genes previously identified in more primitive species.

During the past decade, differential cDNA cloning methods, including *e.g.*, conventional subtractive hybridization (Hla and Maciag, *Biochem. Biophys. Res. Commun.* *167*:637-643 (1990a)), differential polymerase chain reaction (PCR)-oriented hybridization (Hla and Maciag, *J. Biol. Chem.* *265*:9308-9313 (1990b)), and more recently, a modification of the differential display (Zimrin *et al.*, *Biochem. Biophys. Res. Commun.* *213*:630-638 (1995)) were used to identify genes induced during the process of human umbilical vein endothelial cell (HUVEC) differentiation *in vitro*. Very early studies disclosed that HUVEC populations are able to generate capillary-like, lumen-containing structures when introduced into a growth-limited environment *in vitro* (Maciag *et al.*, *J. Cell Biol.* *94*:511-520 (1982)). These studies permitted the identification and characterization of protein components of the extracellular matrix as inducers of this differentiation process, while at the same time defining the capillary-like structures as non-terminally differentiated (Maciag, 1984). Additional experiments have

elucidated the importance of polypeptide cytokines, such as IL-1 (Maier *et al., J. Biol. Chem. 265*:10805-10808 (1990a)), and IFNγ (Friesel *et al., J. Cell Biol. 104*:689-696 (1987)) as inducers of HUVEC differentiation *in vitro*, and ultimately lead to an understanding that the precursor form of IL-1α was responsible for the induction of HUVEC senescence *in vitro* (Maciag *et al., J. Cell Biol. 91*:420-426 (1981); Maier *et al., Science 249*:1570-1574 (1990b)) - the only truly terminal HUVEC phenotype identified to date. Summarized in Figure 1.

Recent research has employed differential cDNA cloning methods, which permits the identification of new and very interesting genes. However, until very recently, establishing their identity did not provide insight into the mechanism of HUVEC differentiation. Current research has focused upon the fibroblast growth factor (FGF) and interleukin (IL)-1 gene families as regulators of the angiogenesis process, both *in vitro* and *in vivo* (Friesel *et al., FASEB J 9*:919-925 (1995); Zimrin *et al., J. Clin. Invest. 97*:1359 (1996)). The human umbilical vein endothelial cell (HUVEC) has proven to be an effective model for studying the signal pathways utilized by FGF-1 to initiate HUVEC migration and growth, the role of IL-1α as an intracellular inhibitor of FGF-1 function and modifier of HUVEC senescence, and the interplay between the FGF and the IL-1 gene families as key effectors of HUVEC differentiation *in vitro*. Such insight has enabled the present inventors to use modern molecular methods to identify a key regulatory ligand-receptor signaling system, which is able to *both* induce capillary endothelial cell migration and repress large vessel endothelial cell migration.

The Jagged/Serrate/Delta-Notch/Lin/Glp signaling system, originally described during the development of *C. elegans* and *Drosophila* as an essential system instrumental in cell fate decisions, has been found to be highly conserved in mammalian cells (Nye and Kopan, *Curr. Biol. 5*:966-969 (1995)). Notch proteins comprise a family of closely-related transmembrane receptors initially identified in embryologic studies in *Drosophila* (Fortini and Artavanis-Tsakonas, *Cell 75*:1245-1247 (1993)). The genes encoding the Notch receptor show a high degree of structural conservation, and contain multiple EGF repeats in their extracellular domains (Coffman *et al., Science 249*:1438-1441 (1990); Ellisen *et al., Cell 66*:649-661 (1991); Weinmaster *et al., Development 113*:199-205 (1991); Weinmaster *et al., Development 116*:931-941 (1992); Franco del Amo *et al., Development 115*:737-744 (1992); Reaume *et al., Dev. Biol. 154*:377-387 (1992); Lardelli and Lendahi, *Mech. Dev. 46*:123-136 (1993); Bierkamp and Campos-Ortega, *Mech. Dev. 43*:87-100 (1993); Lardelli *et al., Exp. Cell Res.*

204:364-372 (1994)). In addition to the 36 EGF repeats within the extracellular domain of Notch 1, there is a cys-rich domain composed of three Notch Lin Glp (NLG) repeats, which is important for ligand function, followed by a cys-poor region between the transmembrane and NLG domain.

The intracellular domain of Notch 1 contains six ankyrin/Cdc10 repeats positioned between two nuclear localization sequences (NLS) (Artavanis-Tsakonas *et al.*, *Science 268*:225-232 (1995)). This motif is found in many functionally diverse proteins (*see e.g.*, Bork, *Proteins 17*:363-374 (1993)), including members of the rel/NF-kB family (Blank *et al.*, *TIBS 17*:135-140 (1992)), and is thought to be responsible for protein-protein interactions. Notch has been shown to interact with a novel ubiquitously distributed cytoplasmic protein deltex through its ankyrin repeats, a domain shown by deletion analysis to be necessary for activity (Matsuno *et al.*, *Development 121*:2633-2644 (1995)).

Carboxy terminal to this region is a polyglutamine-rich domain (OPA) and a pro-glu-ser-thr (PEST) domain which may be involved in signaling protein degradation. There are numerous Notch homologs, including three Notch genes. (The corresponding structures for Lin-12 and Glp-1 are shown in Figure 2.)

Several Notch ligands have been identified in vertebrates, including Delta, Serrate and Jagged. The Notch ligands are also transmembrane proteins, having highly conserved structures. These ligands are known to signal cell fate and pattern formation decisions through the binding to the Lin-12/Notch family of transmembrane receptors (Muskavitch and Hoffmann, *Curr. Top. Dev. Biol. 24*:289-328 (1990); Artavanis-Tsakonas and Simpson, *Trends Genet. 7*:403-408 (1991); Greenwald and Rubin, *Cell 68*:271-281 (1992); Gurdon, *Cell 68*:185-199 (1992); Fortini and Artavanis-Tsakonas, 1993; and Weintraub, *Cell 75*:1241-1244 (1993)). A related protein, the Suppressor of hairless (Su(H)), when co-expressed with Notch in *Drosophila* cells, is sequestered in the cytosol, but is translocated to the nucleus when Notch binds to its ligand Delta (Fortini and Artavanis-Tsakonas, 1993). Studies with constitutively activated Notch proteins missing their extracellular domains have shown that activated Notch suppresses neurogenic and mesodermal differentiation (Coffman *et al.*, *Cell 73*:659-671 (1993); Nye *et al.*, *Development 120*:2421-2430 (1994)).

The Notch signaling pathway (Figure 3), which is apparently activated by Jagged in the endothelial cell, involves cleavage of the intracellular domain by a protease, followed by nuclear

trafficking of the Notch fragment and the interaction of this fragment with the $KBF_2/RBP-J_k$ transcription factor (Jarriault *et al., Nature 377*:355-358 (1995); Kopan *et al., Proc. Natl. Acad. Sci. USA 93*:1683-1688 (1996)), a homolog of the *Drosophila* Suppressor of hairless gene (Schweisguth *et al., Cell 69*:1199-1212 (1992)), a basic helix-loop-helix transcription factor involved in Notch signaling in insects (Jennings *et al., Development 120*:3537-3548 (1994)) and in the mouse (Sasai *et al., Genes Dev. 6*:2620-2634 (1992)). This effector is able to repress the transcriptional activity of other genes encoding transcription factors responsible for entry into the terminal differentiation program (Nye *et al.*, 1994; Kopan *et al., J. Cell. Physiol. 125*:1-9 (1994)).

The Jagged gene encodes a transmembrane protein which is directed to the cell surface by the presence of a signal peptide sequence (Lindsell *et al., Cell 80*:909-917 (1995)). While the intracellular domain contains a sequence with no known homology to intracellular regions of other transmembrane structures, the extracellular region of the ligand contains a cys-rich region, 16 epidermal growth factor (EGF) repeats, and a DSL (Delta Serrate Lag) domain. As shown in Figure 4, the DSL domain as well as the EGF repeats, are found in other genes including the *Drosophila* ligands, Serrate (Baker *et al., Science 250*:1370-13771990; Thomas *et al., Development 111*:749-761 (1991)) and Delta (Kopczynski *et al., Genes Dev. 2*:1723-1735 (1988)), and *C. elegans* genes Apx-1 (Henderson *et al., Development 120*:2913-2924 (1994); Mello *et al., Cell 77*:95-106 (1994)) and Lag-2 (Tax *et al., Nature 368*, 150-154 (1994)).

Nevertheless, until the discovery of the presently disclosed invention, human Jagged remained undefined and the function and relationship, if any, of the human ligand to Notch remained unknown in the art. However, there was a recognized need in the art for a complete understanding of the protein's role in the regulation of cell differentiation and regulation. As disclosed in the present invention, the human Jagged gene has now been cloned, isolated and defined, and the Jagged-Notch role in endothelial cell differentiation and/or migration has been elucidated. In addition, it is presently disclosed that the novel signaling pathway produces disparate effects on the migration of large and small vessel endothelial cells, providing what appears to be the first demonstration of a signaling difference between large and small vessel endothelial cells both in degree and direction. This highlights the potential function of a previously unknown ligand-receptor signaling pathway in the endothelial cell which is

modulated during the migratory phase of angiogenesis. Moreover, the present invention provides an explanation of the previously unresolved phenomenon in which endothelial cells have been shown to reproducibly differentiate into a non-terminal and completely reversible tubular-like cell phenotype *in vitro* (Maciag *et al.*, 1982). Thus, the present invention significantly advances the art, providing not only methods of regulating cell differentiation and angiogenesis, but also teaching a method for preventing the undesirable migration of specific cell types into large blood vessels following angioplastic surgery to control restenosis.

SUMMARY OF THE INVENTION

The present invention relates to a novel discovery of human Jagged and of the role of Jagged-Notch in endothelial cell migration and/or differentiation, and to the determination that the signaling pathway produces disparate effects on the migration of large and small vessel endothelial cells.

The invention provides a substantially purified Jagged protein, *i.e.*, a peptide free of the proteins with which it is normally associated, particularly a human Jagged protein; it also provides a functionally equivalent derivative, or allelic or species variant thereof. It further provides a peptide which has an amino acid sequence corresponding to SEQ ID NO:1. Moreover, the invention provides a protein which is characterized by the ability to bind to Notch.

The invention provides a substantially purified nucleic acid molecule encoding a Jagged protein, particularly a human Jagged protein; it also provides a nucleic acid molecule or DNA segment thereof encoding a functionally equivalent derivative, or allelic or species variant thereof. It further provides a nucleic acid sequence having a sequence corresponding to SEQ ID NO:1. Moreover, the invention provides a nucleic acid sequence encoding a human protein which is characterized by the ability to bind to Notch.

In addition, the invention provides a recombinant molecule comprising a vector and the nucleic acid sequence or segment thereof encoding the Jagged protein or functional portion thereof, particularly the human Jagged protein. It also provide a host cell comprising the recombinant molecule comprising a vector and the nucleic acid sequence or segment thereof encoding the Jagged protein or functional portion thereof. The invention further provides the

expression product of the recombinant molecule comprising a vector and the nucleic acid sequence encoding the Jagged protein.

Further, the invention provides a substantially purified, single-stranded, nucleic acid molecule comprising the antisense strand of the Jagged cDNA (γ-Jagged), particularly of the cDNA for the human Jagged protein; it also provides DNA segments which if read in the sense direction would encode a functionally equivalent derivative, or allelic or species variant thereof. It also provides the nucleic acid molecule comprising the antisense nucleotide sequence corresponding to the antisense strand of SEQ ID NO:1. Moreover, the invention provides an antisense molecule which is characterized by the ability to bind to Jagged, or a functionally equivalent derivative, or allelic or species variant thereof.

The invention also provides the polypeptide encoded by the nucleic acid molecule comprising the antisense strand of the Jagged cDNA (γ-Jagged), particularly of the cDNA for the human Jagged protein. It further provides the polypeptide encoded by the antisense Jagged molecule, wherein the polypeptide has a binding affinity to, and inhibits the activity of Jagged.

In addition, the invention provides an antibody having a binding affinity to Jagged, or a unique portion thereof.

It also provides a secondary antibody having a binding affinity to anti-Jagged, or a unique portion thereof.

The invention provides a method of decreasing the migration of endothelial cells to a site on a micro-diameter blood vessel, comprising delivering a Jagged protein, or a functionally equivalent derivative, or allelic or species variant thereof, or a secondary anti-Jagged antibody to a site from which the endothelial cells have been removed, damaged or substantially reduced. It also provides a method of decreasing the migration of endothelial cells, particularly human endothelial cells, to a site on a macro-diameter blood vessel, comprising delivering an antisense Jagged molecule (γ-Jagged) or a Jagged antibody to a site from which the endothelial cells have been removed, damaged or substantially reduced.

The invention provides a method of increasing the migration of endothelial cells, particularly human endothelial cells, to a site on a macro-diameter blood vessel, comprising delivering a Jagged protein, or a functionally equivalent derivative, or allelic or species variant thereof, or a secondary anti-Jagged antibody to a site from which the endothelial cells have been removed, damaged or substantially reduced. It also provides a method of increasing the

migration of endothelial cells, particularly human endothelial cells, to a site on a micro-diameter blood vessel, comprising delivering an antisense Jagged molecule (γ-Jagged) or a Jagged antibody to a site from which the endothelial cells have been removed, damaged or substantially reduced.

Moreover, the invention provides a method of decreasing the migration of smooth muscle cells, particularly human smooth muscle cells, to a site on a macro-diameter blood vessel, comprising delivering an antisense Jagged molecule (γ-Jagged) or a Jagged antibody to a site from which the endothelial cells have been removed, damaged or substantially reduced.

The invention also provides a pharmaceutical composition comprising a therapeutically effective amount of a Jagged protein, or functionally equivalent derivative, or allelic or species variant thereof, particularly a human Jagged protein; and a pharmaceutically acceptable carrier. Also provided is a pharmaceutical composition comprising a therapeutically effective amount of a Jagged nucleic acid, or functionally equivalent derivative, or allelic or species variant thereof, particularly a human Jagged nucleic acid; and a pharmaceutically acceptable carrier.

In addition, the invention provides a pharmaceutical composition comprising a therapeutically effective amount of a Jagged antibody, or functionally equivalent derivative, or allelic or species variant thereof; and a pharmaceutically acceptable carrier. Also provided is a pharmaceutical composition comprising a therapeutically effective amount of a Jagged antisense molecule, or functionally equivalent derivative, or allelic or species variant thereof; and a pharmaceutically acceptable carrier. Further provided is a pharmaceutical composition comprising a therapeutically effective amount of an anti-Jagged antibody, or functionally equivalent derivative, or allelic or species variant thereof; and a pharmaceutically acceptable carrier.

The invention also provides a method of preventing or treating a disease or condition in a subject comprising administering to a subject in need of such prevention or treatment a therapeutically effective amount of a molecule which antagonizes, inhibits or prevents the function of the Notch protein; or comprising administering a therapeutically effective amount of a molecule which agonizes, enhances or stimulates the function of the Notch protein. It further provides a method of preventing or treating a disease or condition in a subject comprising administering to a subject in need of such prevention or treatment a therapeutically effective amount of a molecule which antagonizes, inhibits or prevents the function of the

Jagged protein; or comprising administering a therapeutically effective amount of a molecule which agonizes, enhances or stimulates the function of the Jagged protein.

In addition, the invention provides a method of inhibiting or preventing angiogenesis in a subject comprising administering to a subject in need of such inhibition or prevention a therapeutically effective amount of Jagged or a Jagged agonist. The angiogenesis being inhibited or prevented comprises solid tumor angiogenesis, rheumatoid arthritic angiogenesis, inflammatory angiogenesis, and the like. The invention also provides a method of inhibiting or preventing restenosis of the lumen of a blood vessel, by repressing angiogenesis from the vaso vasorum, and by promoting large vessel endothelial cell migration to repair the lumen of a large blood vessel. These methods of inhibiting or preventing angiogenesis are provided *in vivo* and/or *in vitro*. Also provided are Jagged agonists comprising agents which promote the expression of Jagged, including fibrin and functional derivatives thereof and pharmacologically acceptable chemicals, and γ-idiotypic Jagged antibodies.

Moreover, the invention provides a method of promoting or enhancing angiogenesis in a subject comprising administering to a subject in need of such promotion or enhancement a therapeutically effective amount of anti-Jagged or a Jagged antagonist. The angiogenesis being promoted or enhanced comprises wound or injury repair angiogenesis, such as that which occurs in a wound or injury caused by surgery, trauma and/or disease or condition, including diabetes-related wounds or injuries. These methods of promoting or enhancing angiogenesis are provided *in vivo* and/or *in vitro*. Also provided are Jagged antagonists comprising Jagged antibodies, anti-sense Jagged, Jagged mutants and pharmacologically acceptable chemicals.

The invention further provides a method for affecting cell differentiation of cells comprising the mesoderm, endoderm, ectoderm and/or neuroderm. Also provided is a method for affecting cell differentiation of cells, wherein the cell types affected comprise hematopoietic stem cells, epithelial cells, vascular smooth muscle cells and dendritic cells.

In addition, the invention provides a pharmaceutical composition used in any of the previously disclosed methods.

Additional objects, advantages and novel features of the invention will be set forth in part in the description which follows, and in part will become apparent to those skilled in the art on examination of the following, or may be learned by practice of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Illustration of the phenotypic alterations of HUVEC by cytokines. Early studies demonstrated that HUVEC populations are able to generate capillary-like, lumen-containing structures when introduced into a growth-limited environment *in vitro*. However, exposure of an HUVEC population to polypeptide cytokines, such as IL-1 and IFNγ, as inducers of HUVEC differentiation *in vitro*, lead to an understanding that the precursor form of IL-1α was responsible for the induction of HUVEC senescence *in vitro*, the only truly terminal HUVEC phenotype identified to date. (PD = population doubling).

Figure 2. Illustration of the domain structure of the Notch receptor family. (Numbers refer to the number of EGF repeats in the extracellular domain.) As indicated in this chart, in addition to the 36 EGF repeats within the extracellular domain of Notch 1, there is a cys-rich domain composed of three Notch-Lin-Glp (NLG) repeats, followed by a cys-poor region between the transmembrane and NLG domain. The intracellular domain of Notch 1 contains six ankyrin/Cdc10 repeats positioned between two nuclear localization sequences (NLS). In the carboxy-terminal direction from this region is a polyglutamine-rich domain (OPA) and a pro-glu-ser-thr (PEST) domain. Comparable structures are shown for Lin-12 and Glp-1.

Figure 3. The Notch signaling pathway. The components of the Notch signaling pathway are illustrated, using the myoblast as an example. The Notch signaling pathway, when activated by Jagged in the endothelial cell, involves cleavage of the intracellular domain by a protease, nuclear trafficking of the Notch fragment and the interaction of this fragment with the KBF_2/RBP-Jk transcription factor, a homolog of the *Drosophila* Suppressor of Hairless (Su(H)) gene, which is a basic helix-loop-helix transcription factor involved in Notch signaling.

Figure 4. Illustration of the domain structure of the Notch ligand family. (Numbers refer to the number of EGF repeats in the extracellular domain.) As indicated in this chart, although the intracellular domain of the Jagged gene contains a sequence with no known homology to intracellular regions of other transmembrane structures, the extracellular region of the gene contains a cys-rich region, 16 epidermal growth factor (EGF) repeats, and a Delta-Serrate-Lag (DSL) domain, typical of comparable regions found in other genes including the *Drosophila* ligands, Serrate and Delta, and the *C. elegans* genes, Apx-1 and Lag-2.

Figure 5. RT-PCR analysis of steady-state levels of Jagged, Notch 1 and Notch 2 transcripts in HUVEC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a positive control.

Figure 6. Graphic representation of the effect of the antisense Jagged oligonucleotide on BMBC sprout formation, as compared with the effect on three control oligomers, a Jagged sense oligonucleotide, a 3' antisense Jagged oligomer, and a mutated 5' antisense Jagged oligomer.

Figures 7A and 7B. Line graphs showing the effect of the antisense Jagged oligonucleotide on bovine endothelial cell migration. The effect on bovine microvascular endothelial cells (BMEC) is shown on Figure 7A; the effect on bovine aorta endothelial cells (BAEC) is shown on Figure 7B.

DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

Definitions

In the description that follows, a number of terms used in the claims as well as in recombinant DNA technology are extensively utilized. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such claims, the following definitions are provided.

DNA segment. A DNA segment, as is generally understood and as used herein, refers to a molecule comprising a linear stretch of nucleotides wherein the nucleotides are present in a sequence that encodes, through the genetic code, a molecule comprising a linear sequence of amino acid residues that is referred to as a protein, a protein fragment, or a polypeptide.

Gene. A DNA sequence related to a single polypeptide chain or protein, and as used herein includes the 5' and 3' ends. The polypeptide can be encoded by a full-length sequence or any portion of the coding sequence, so long as the functional activity of the protein is retained.

A "complimentary DNA" or "cDNA" gene includes recombinant genes synthesized by reverse transcription of messenger RNA ("mRNA") lacking intervening sequences (introns).

Structural gene. A DNA sequence that is transcribed into mRNA that is then translated into a sequence of amino acids characteristic of a specific polypeptide. Typically the first nucleotide of the first translated codon is numbered +1, and the nucleotides are numbered

consecutively with positive integers through the translated region of the structural gene and into the 3' untranslated region. The numbering of the nucleotides in the promoter and regulatory region 5' to the translated region proceeds consecutively with negative integers with the 5' nucleotide next to the first translated nucleotide being numbered -1.

Gel electrophoresis. To detect determine the size of particular DNA fragments, the most common technique (although not the only one) is agarose gel electrophoresis, which is based on the principle that DNA molecules migrate through the gel as though it were a sieve that retards the movement of the largest molecules to the greatest extend, and the movement of the smallest molecules to the least extent. The fractionated molecules can be visualized by staining, permitting the DNA fragments of a genome to be visualized. However, most genomes, including the human genome, contain too many DNA sequences to produce an easily visualized pattern. Thus, a methodology referred as "Southern hybridization" (or "blotting") is used to visualize small subsets of fragments. By this procedure the fractionated DNA is physically transferred onto nitrocellulose filter paper or another appropriate surface using recognized methods. Note that RNA fragments can be similarly visualized by the "northern blot" process.

Nucleic acid hybridization. This process depends on the principle that two single-stranded molecules that have complimentary base sequences will reform into the thermodynamically favored double-stranded configuration ("reanneal") if they are mixed in solution under the proper conditions. The reannealling process will occur even if one of the single strands is immobilized.

Hybridization probe. To visualize a particular DNA sequence in the hybridization procedure, a labeled DNA molecule or hybridization probe is reacted to the fractionated nucleic acid bound to the nitrocellulose filter. The areas on the filter that carry nucleic acid sequences complementary to the labeled DNA probe become labeled themselves as a consequence of the reannealing reaction. The areas of the filter that exhibit such labeling are visualized. The hybridization probe is generally produced by molecular cloning of a specific DNA sequence.

Oligonucleotide or Oligomer. A molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three. Its exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide. An oligonucleotide may be derived synthetically or by cloning.

Sequence Amplification. A method for generating large amounts of a target sequence. In general, one or more amplification primers are annealed to a nucleic acid sequence. Using appropriate enzymes, sequences found adjacent to, or in between the primers are amplified.

Amplification primer. An oligonucleotide which is capable of annealing adjacent to a target sequence and serving as an initiation point for DNA synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is initiated.

Vector. A plasmid or phage DNA or other DNA sequence into which DNA may be inserted to be cloned. The vector may replicate autonomously in a host cell, and may be further characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion and into which DNA may be inserted. The vector may further contain a marker suitable for use in the identification of cells transformed with the vector. The words "cloning vehicle" are sometimes used for "vector."

Expression. Expression is the process by which a structural gene produces a polypeptide. It involves transcription of the gene into mRNA, and the translation of such mRNA into polypeptide(s).

Expression vector. A vector or vehicle similar to a cloning vector but which is capable of expressing a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (*i.e.*, operably linked to) certain control sequences such as promoter sequences. Expression control sequences will vary depending on whether the vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host and may additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

Functional derivative. A "functional derivative" of a sequence, either protein or nucleic acid, is a molecule that possesses a biological activity (either functional or structural) that is substantially similar to a biological activity of the protein or nucleic acid sequence. A functional derivative of a protein may or may not contain post-translational modifications such as covalently linked carbohydrate, depending on the necessity of such modifications for the performance of a specific function. The term "functional derivative" is intended to include the "fragments," "segments," "variants," "analogs," or "chemical derivatives" of a molecule.

As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, and the like. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, and the like. Moieties capable of mediating such effects are disclosed in *Remington's Pharmaceutical Sciences* (1980). Procedures for coupling such moieties to a molecule are well known in the art.

Variant. A "variant" or "allelic or species variant" of a protein or nucleic acid is meant to refer to a molecule substantially similar in structure and biological activity to either the protein or nucleic acid. Thus, provided that two molecules possess a common activity and may substitute for each other, they are considered variants as that term is used herein even if the composition or secondary, tertiary, or quaternary structure of one of the molecules is not identical to that found in the other, or if the amino acid or nucleotide sequence is not identical.

Substantially pure. A "substantially pure" protein or nucleic acid is a protein or nucleic acid preparation that is generally lacking in other cellular components with which it is normally associated *in vivo*.

Ligand. "Ligand" refers to any protein or proteins that may interact with a receptor binding domain, thus having a "binding affinity" for such domain. Ligands may be soluble or membrane bound, and they may be a naturally occurring protein, or synthetically or recombinantly produced. The ligand may also be a nonprotein molecule that acts as ligand when it interacts with the receptor binding domain. Interactions between the ligand and receptor binding domain include, but are not limited to, any covalent or non-covalent interactions. The receptor binding domain is any region of the receptor molecule, *e.g.* Notch, that interacts directly or indirectly with the ligand, *e.g.*, Jagged. If the Notch-Jagged interaction acts as an on-off switch, Jagged may provide the receptor binding domain, and Notch or a component produced as a result of the Notch-Jagged interaction may act as the ligand.

"Antisense nucleic acid sequence," "antisense sequence," "antisense DNA molecule" or "antisense gene" refer to pseudogenes which are constructed by reversing the orientation of the gene with regard to its promoter, so that the antisense strand is transcribed. The term also refers to the antisense strand of RNA or of cDNA which compliments the strand of DNA encoding the protein or peptide of interest. In either case, when introduced into a cell under

the control of a promoter, the anti-sense nucleic acid sequence inhibits the synthesis of the protein of interest from the endogenous gene. The inhibition appears to depend on the formation of an RNA-RNA or cDNA-RNA duplex in the nucleus or in the cytoplasm. Thus, if the antisense gene is stably introduced into a cultured cell, the normal processing and/or transport is affected if a sense-antisense duplex forms in the nucleus; or if antisense RNA is introduced into the cytoplasm of the cell, the expression or translation of the endogenous product is inhibited. Such antisense nucleic acid sequences may further include modifications which could affect the biological activity of the antisense molecule, or its manner or rate of expression. Such modifications may also include, *e.g.*. mutations, insertions, deletions, or substitutions of one or more nucleotides that do not affect the function of the antisense molecule, but which may affect intracellular localization. Modifications include, but are not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxmethyl uracil, 5-carboxyhydroxmethyl-2-thiouridine, 5-carboxymethylaminomethyl uracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentyladenine, 1-methylguanine, 1-methyinosine, 2,2dimthylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methylaminomethyl-2-thioracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methyluracil, 2-methylthio-N6-isopentenyladenine, uracil-5oxyacetic acid, wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, 5-methy-2-thiouracil, 3-(3-amino-3-N-2-carboxpropyl) uracil, and 2,6-diaminopurine.

The nucleic acid sequence may determine an uninterrupted antisense RNA sequence or it may include one or more introns. The antisense Jagged molecule(s) of the present invention are referred to as γ-Jagged.

Steady-state level. The term refers to a stable condition that does not change over time, or the state in which change in one direction or production of a component is continually balanced by a compensatory change in another.

Preferred Embodiments

Angiogenesis, or the formation of new blood vessels, plays a central role in a number of physiologic and pathologic conditions, including placental development, wound healing,

rheumatoid arthritis, diabetic retinopathy and solid tumor growth and metastasis. Endothelial cells comprise a monolayer lining the luminal surface of all blood vessels, thereby playing a central role in this process. *In vitro* populations of endothelial cells isolated from both large vessels and microvessels can be induced to mimic this differentiation process by forming a capillary-like network. Three-dimensional fibrin gels have been used to mimic angiogenesis, as an *in vitro* corollary of the *in vivo* phenomenon since endothelial cells invade blood clots in the process of wound repair.

Cellular differentiation is a well documented process *in vitro*, generally requiring a transcriptional component for induction. However, in contrast to most cell types, endothelial cell differentiation has been shown to be reversible. Digestion of the endothelial cellular networks formed *in vitro*, and subsequent culture of the cells in the presence of FGF-1 causes them to revert to an undifferentiated phenotype (*see, e.g.*, Maciag *et al., J. Cell Biol. 94*:511-520 (1982)). However, endothelial cell differentiation has also been shown to have a transcriptional basis. Endothelial cell (HUVEC) organization into a cellular network has been shown to be associated with an increase in the transcript encoding fibronectin, and a decrease in the transcript encoding *sis*, which reverses when the cellular network is digested with proteases and the cells revert to the proliferative phenotype (*see e.g.*, Jaye *et al., Science 228*:882-885 (1985)).

HUVEC are capable of two different behaviors, both of which are termed "differentiation." The first is the formation of a two dimensional network involving cell elongation, anastomosis and branching that does not require transcription and translation, but requires post-translational modification. The second is a more complex three-dimensional process resulting in a capillary network containing lumens, which Zimrin *et al.* (1995) have shown requires both transcriptional and post-translational events. In addition, Zimrin *et al.* (1995) has defined the modified differential display technique as applied to endothelial cells and demonstrated that it is a very useful method of isolating transcripts which are differentially expressed as endothelial cells differentiate.

Thus, in the present invention, using a modification of the differential display method, the human homolog of the Jagged ligand for the Notch receptor has been isolated from human umbilical vein endothelial cells (HUVEC) invading a fibrin gel. The addition of an antisense Jagged oligonucleotide to bovine microvascular endothelial cells on collagen resulted in a

marked increase in their invasion into the collagen gel in response to FGF-2. However, while the antisense Jagged oligonucleotide of the present invention was also able to increase the migration of bovine microvascular endothelial cells on fibronectin, the oligonucleotide significantly decreased the migration of bovine endothelial cells derived from the aorta, suggesting a divergence in the mechanism utilized by two different endothelial cell populations to respond to the Notch signaling system.

The distinction between microvascular and large vessel endothelium is well recognized as a part of the heterogeneity of the vascular endothelium in general and this is reflected in the properties of endothelial cells from different sources in cell culture (Carson and Haudenschild, *In Vitro 22*:344-354 (1986)), and in organ-specific expression of different adhesion molecules, cell surface glycoproteins and lectin-binding sites (Gumkowski *et al., Blood Vessels 24*:11 (1987)).

Briefly, to identify the molecular events necessary in the process of angiogenesis, a modified differential display procedure was used to isolate messages that were differentially expressed in HUVEC plated on fibrin in the presence of FGF-1 over the course of 24 hours. As described in Example 2, one of the cDNAs that was amplified at 2 hours, and which was found to be highly homologous to the rat Jagged transcript was identified as an isolate of the human Jagged homolog. The putative protein sequence of the present invention includes a signal peptide, a DSL domain shared by the Notch ligands Delta, Serrate, LAG-2 and APX-1, 16 tandem epidermal growth factor-like repeats, a cysteine-rich region, a transmembrane domain and a 125 base pair cytoplasmic tail. The 5' end of the sequence of the human Jagged isolate corresponds to position 417 of the rat sequence, at the eleventh codon of the predicted 21 residue signal peptide.

To investigate the role of Jagged and Notch in endothelial cell behavior, reverse transcription and polymerase chain reaction amplification (RT-PCR) was used to evaluate the steady-state message levels of Jagged and two related Notch proteins, human TAN-1 and human Notch group protein, in human endothelial cells on fibrin (Figure 5). Although the Jagged message was found to be up-regulated in populations of HUVEC exposed to fibrin at the 3 hour timepoint, the message levels of the two Notch proteins was not changed over the course of 24 hours. Thus, it is shown in the present invention that the human endothelial cell population is capable of expressing both the Jagged ligand and the Notch receptor, indicating

that the human endothelial cell is completing an autocrine signal using the Notch signal transduction pathway. The data do not distinguish, however, between a homogenous population expressing both Notch and Jagged proteins, or heterogeneous subpopulations of endothelial cells that display Notch, Jagged, both or neither protein.

Therefore, to delineate a functional role for Jagged, an antisense Jagged oligonucleotide was designed in the present invention, which encompassed the Kozak consensus region, the ATG start codon and the next three codons of the rat Jagged cDNA sequence. Similar strategies have previously proved useful as a means of repressing the translational efficiency of a wide variety of transcripts *in vitro* (*see*, Scanlon *et al.*, *FASEB J.* *9*:1288-1296 (1995); Maier *et al.*, 1990b).

Because endothelial cell migration is an important component of angiogenesis, endothelial cell behavior was evaluated under conditions of sprout formation (Montesano and Orci, *Cell 42*:469-477 (1985)) and migration (Sato and Rifkin, *J. Cell Biol. 107*:1199-1205 (1988)). The addition of the oligonucleotide to bovine microvascular endothelial cells plated on collagen at varying concentrations resulted in an oligonucleotide-induced dose-dependent increase in the total length of sprout formation observed in response to the addition of FGF-2 (Figure 6). The addition of several control oligonucleotides, including a sense oligonucleotide covering the same sequence, a 5' antisense oligonucleotide with every third base mutated, and a random oligonucleotide, had no effect on the total length of sprout formation (Figure 6). Thus, the addition of the antisense Jagged oligonucleotide significantly enhanced endothelial cell sprout formation beyond the level achieved by FGF-2.

These data were unusual since endothelial cell sprout formation requires cell migration as a component, and the Jagged cDNA had been isolated from a human endothelial cell system where migration into the fibrin clot also occurs. Consequently, the effect of the antisense Jagged oligonucleotide was studied on capillary and large vessel endothelial cell migration, respectively. It was found that while a bovine microvascular endothelial cell population exhibited a significant dose-dependent increase in their migration in the presence of the Jagged antisense oligonucleotide (Figure 7A), the migration of bovine aorta endothelial cells was significantly attenuated in a dose-dependent fashion by the antisense Jagged oligonucleotide (Figure 7B). Thus, the ability of Jagged-Notch signaling to modify endothelial cells was dependent upon the anatomic source of the endothelial cells.

Since the endothelial cells studied were from both large and small vessels responded to the antisense Jagged oligonucleotide in a disparate manner, and both cellular populations are likely to express the Notch receptor, the difference in their response to the Jagged antisense oligonucleotide indicates for the first time that there are differences between large and small vessels in the Notch signaling pathway. Although it has been documented that cells isolated from small vessels are able to undergo the phenotypic changes characteristic of capillary formation more readily than endothelial cells isolated from large vessels (Ingber and Folkman, *J. Cell Biol. 109*:317-330 (1989)), the novel response to the Jagged antisense oligonucleotide disclosed in the present invention represents the first demonstration of an effect not only different in degree but also in direction.

The present embodiments further demonstrate that the addition of exogenous Jagged (or enhanced expression of Jagged) produces an effect opposite to that seen in Examples 5-7. In other words, the addition or increased expression of Jagged will decrease the migration and invasion of microvascular cells from the vaso vasorum, and increase or stimulate the migration of large vessel endothelial cells.

The clinical importance of the disparate effect of the Jagged-Notch signaling pathway on the macro- and micro-diameter blood vessels is significant, offering a solution to many aspects of vascular pathophysiology. For example, the morbidity and mortality from hypertension is clearly based on the disease of the large vessels (atherosclerosis and stroke), but in the major forms of hypertension, the actual cause for elevated blood pressure lies in the peripheral vascular beds (arterioles and microvasculature) (Chobanian *et al.*, *Hypertension 8*:15-21 (1986)). The presently defined compositions and methods may resolve the previously unanswered question of how hypertension could be directly related to the aortic intima and atherosclerosis, and vice versa, how known atherogenic risk factors could affect the microvascular endothelium (Chan *et al.*, *Microvasc. Res. 18*:353-369 (1979)).

Moreover, the presently embodied compositions and methods are useful for the modification of a post-angioplastic situation, when one or more large coronary vessel have been stripped of their endothelial cell lining. One of the most serious complications limiting the value of the angioplastic procedure is the occurrence of restenosis or the rapid migration and proliferation of smooth muscle cells, monocytes/macrophages, platelets, and endothelium at the wound site resulting in a reocclusion of the vessel that may be more extensive than before

treatment (*see* numerous review articles on the subject, *e.g.*, Schwartz *et al.*, *Atherosclerosis* *1*:107-161 (1981)). However, treating the wounded or injured area with a therapeutic amount of additional recombinant Jagged protein, or a functionally equivalent drug or protein having the ability to signal Notch, will prevent or inhibit reocclusion by *increasing* the migration of the large vessel endothelial cells on the borders of the lesion into the denuded area to cover the lesion, while also *decreasing* emergence of the micro-vascular cells (smooth muscle, endothelial, macrophage, etc) from the vaso vasorum and providing the nutrient microvessels or sprouts to supply the proliferating smooth muscle cells.

In a preferred embodiment, the present invention provides highly purified Jagged protein. As used herein, a protein is said to be highly purified if the protein possesses a specific activity that is greater than that found in whole cell extracts containing the protein.

Any eukaryotic organism can be used as a source of Jagged, or the genes encoding same, as long as the source organism naturally contains the ligand or its equivalent. As used herein, "source organism" refers to the original organism from which the amino acid or DNA sequence is derived, regardless of the organism the ligand is expressed in or ultimately isolated from. For example, a human is said to be the "source organism" of Jagged expressed by an insect expression system as long as the amino acid sequence is that of human Jagged. The most preferred source organism is human.

A variety of methodologies known in the art can be utilized to obtain the Jagged proteins of the present invention. In one embodiment, the Jagged is purified from tissues or cells which naturally produce it, such as HUVEC. One skilled in the art can readily follow known methods for isolating proteins in order to obtain the Jagged protein. These include, but are not limited to, immunochromotography, size-exclusion chromatography, ion-exchange chromatography, affinity chromatography, HPLC, and the methods set forth by example in the present disclosure. One skilled in the art can readily adapt known purification schemes to delete certain steps or to incorporate additional purification procedures.

In another embodiment, the ligand is purified from cells which have been altered to express the desired protein. As used herein, a cell is said to be "altered to express a desired protein" when the cell, through genetic manipulation, is made to produce a protein which it normally does not produce, or which the cell normally produces at low levels. One skilled in the art can readily adapt procedures for introducing and expressing either genomic or cDNA

sequences into either eukaryotic or prokaryotic cells, in order to generate a cell which produces the desired protein.

There are a variety of source organisms for DNA encoding the desired protein. The more preferred source is the endothelial cell. The most preferred source is the human endothelial cell. The embodied methods are readily adapted to use of an HUVEC population as a model to be evaluated in comparison with HU artery (A) EC and human cells obtained from other anatomic sites. These include human adipose-derived microvascular endothelial cells (HMEC), human dermis-derived capillary endothelial cells (HCEC) and human saphenous vein (HSVEC) and artery (HSAEC). Many human endothelial cell populations are readily available from commercial (HMEC and HCEC) and academic sources (HSVEC and HSAEC; Dr. Michael Watkins, Dept. of Surgery, Boston University, and HUAEC; Dr. Victor van Hinsbergh, Gabius Institute, Netherlands).

In yet another embodiment, since probes are available which are capable of hybridizing to Jagged, DNA sequences encoding the desired nucleic acid sequence encoding the protein of interest can be obtained by routine hybridization and selection from any host which possesses these receptors. A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene sequence expression. The precise nature of the regulatory regions needed for gene sequence expression may vary from organism to organism, but shall in general include a promoter region which, in prokaryotes, contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which, when transcribed into RNA, will signal the initiation of protein synthesis. Such regions will normally include those 5′-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like.

If desired, the non-coding region 3′ to the gene sequence encoding Jagged may be obtained by the above-described methods. This region may be retained for its transcriptional termination regulatory sequences, such as termination and polyadenylation. Thus, by retaining the 3′-region naturally contiguous to the DNA sequence encoding Jagged, the transcriptional

termination signals may be provided. Where the transcriptional termination signals are not satisfactorily functional in the expression host cell, then a 3' region functional in the host cell may be substituted.

Two DNA sequences (such as a promoter region sequence and the Jagged encoding sequence) are said to be operably linked if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region sequence to direct the transcription of the Jagged gene sequence, or (3) interfere with the ability of the Jagged gene sequence to be transcribed by the promoter region sequence. Thus, a promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence. To express Jagged, transcriptional and translational signals recognized by an appropriate host are necessary.

In another embodiment, the nucleic acids sequences of the present invention are under controlled expression by the animal or human patient. In the alternative, the nucleic acids sequences are administered to the patient in need of gene therapy, intravenously, intramuscularly, subcutaneously, enterally, topically, parenterally or surgically. When administering the nucleic acids by injection, the administration may be by continuous administration, or by single or multiple administrations. The gene therapy is intended to be provided to the recipient mammal in a "pharmacologically or pharmaceutically acceptable form" in an amount sufficient to "therapeutically effective." The nuceic acid is said to be in "pharmaceutically or pharmacologically acceptable form" if its administration can be tolerated by a recipient patient. An amount is said to be "therapeutically effective" (also referred to here and elsewhere as "an effective amount") if the dosage, route of administration, etc., of the agent are sufficient to affect a response to Jagged. The nucleic acid is considered to be in "pharmaceutically or pharmacologically acceptable form" if its administration can be tolerated by a recipient patient.

The present invention further encompasses the expression of the Jagged protein (or a functional derivative thereof) in either prokaryotic or eukaryotic cells. Preferred prokaryotic hosts include bacteria such as *E. coli*, *Bacillus*, *Streptomyces*, *Pseudomonas*, *Salmonella*, *Serratia*, etc. Under such conditions, the Jagged will not be glycosylated. The prokaryotic host must be compatible with the replicon and control sequences in the expression plasmid.

However, prokaryotic systems may not prove efficacious for the expression of a soluble Jagged ligand, since the protein of interest contains 1048 residues encompassing residue 22 (after the signal sequence) to residue 1069 (prior to the transmembrane domain). While prokaryotic expression systems, *e.g.*, pET3c, have been used to express high molecular weight proteins, such as a biologically active (molecular weight (M_r) ~118 kDa) FGF-1:β-galactosidase chimera (Shi et al., submitted to *J. Biol. Chem.*, 1996), successful folding and disulfide bond formation for the multiple EGF repeats (three disulfide bonds per EGF repeat) in the Jagged sequence may be difficult to accomplish in bacteria.

Nevertheless, to express Jagged (or a functional derivative thereof) in a prokaryotic cell, it is necessary to operably link the Jagged coding sequence to a functional prokaryotic promoter. Such promoters may be either constitutive or, more preferably, regulatable (*i.e.*, inducible or derepressible). Examples of constitutive promoters include the *int* promoter of bacteriophage λ, the *bla* promoter of the β-lactamase gene sequence of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene sequence of pPR325, etc. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage λ (P_L and P_R), the *trp, recA, lacZ, lacI*, and *gal* promoters of *E. coli*, the α-amylase (Ulmanen, I., *et al., J. Bacteriol. 162*:176-182 (1985)) and the ς-28-specific promoters of *B. subtilis* (Gilman, M.Z., *et al., Gene sequence 32*:11-20 (1984)), the promoters of the bacteriophages of *Bacillus* (Gryczan, T.J., In: *The Molecular Biology of the Bacilli*, Academic Press, Inc., NY (1982)), and *Streptomyces* promoters (Ward, J.M., *et al., Mol. Gen. Genet. 203*:468-478 (1986)). *See also* reviews by Glick, B.R., (*J. Ind. Microbiol. 1*:277-282 (1987)); Cenatiempo, Y. (*Biochimie 68*:505-516 (1986)); and Gottesman, S. (*Ann. Rev. Genet. 18*:415-442 (1984)).

Proper expression in a prokaryotic cell also requires the presence of a ribosome binding site upstream of the gene sequence-encoding sequence. Such ribosome binding sites are disclosed, for example, by Gold, L., *et al.* (*Ann. Rev. Microbiol. 35*:365-404 (1981)).

Preferred eukaryotic hosts include yeast, fungi, insect cells, mammalian cells, either *in vivo* or in tissue culture. Mammalian cells which may be useful as hosts include HeLa cells, cells of fibroblast origin such as VERO or CHO-K1, or cells of lymphoid origin, such as the hybridoma SP2/O-AG14 or the myeloma P3x63Sg8, and their derivatives. Preferred mammalian host cells include SP2/0 and J558L, as well as neuroblastoma cell lines such as IMR 332 that may provide better capacities for correct post-translational processing.

For a mammalian host, several possible vector systems are available for the expression of Jagged. A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, Simian virus, or the like, where the regulatory signals are associated with a particular gene sequence which has a high level of expression. Alternatively, promoters from mammalian expression products, such as actin, collagen, myosin, etc., may be employed. Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the gene sequences can be modulated. Of interest are regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or are subject to chemical (such as metabolite) regulation.

Yeast expression systems can also carry out post-translational peptide modifications. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene sequence products and secretes peptides bearing leader sequences (*i.e.*, pre-peptides). Any of a series of yeast gene sequence expression systems incorporating promoter and termination elements from the actively expressed gene sequences coding for glycolytic enzymes produced in large quantities when yeast are grown in mediums rich in glucose can be utilized. Known glycolytic gene sequences can also provide very efficient transcriptional control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase gene sequence can be utilized.

The more preferred host for a protein the size of Jagged is insect cells, for example the *Drosophila* larvae. Using insect cells as hosts, the *Drosophila* alcohol dehydrogenase promoter can be used (*see, e.g.*, Rubin, G.M., *Science 240*:1453-1459 (1988)).

The baculovirus insect cell expression system is the most preferred system for expressing the soluble Jagged construct (residues 1-1069) as a carboxy-terminal triple tandem myc-epitope repeat:glutathione-S-transferase (GST) fusion protein chimera, using conventional PCR methods (Zhan *et al., J. Biol. Chem. 269*:20221-20224 (1994)). These include the use of recombinant circle PCR to synthesize the soluble Jagged-Myc-GST construct (sJMG), the preparation and expression of the recombinant virus, AcNPV-GsJ in Sf9 cells (Summers and Smith (1988) A Manual of Methods for Baculovirus Vectors and Insect Culture Procedures

(Texas Experimental Station Bulletin #1555)), the use of GST affinity chromatography (Zhan *et al.*, 1994) and reversed phase or ion exchange HPLC to purify the recombinant protein from Sf9 cell lysates and Myc immunoblot analysis to monitor the purification and assess the purity of the sJMG protein.

The sJMG construct may not only prove to be valuable for the baculovirus expression system, but also as a construct for the expression of a secreted and soluble extracellular Jagged ligand in mammalian cells for implantation *in vivo*. Thus, the sJM construct - lacking the GST fusion domain - may be inserted into the pMEXneo vector and stable NIH 3T3 cell transfectants obtained following selection with G418 as described (Zhan *et al.*, *Biochem. Biophys. Res. Commun. 188*:982-991 (1992). Moreover, baculovirus vectors can be engineered to express large amounts of Jagged in insect cells (Jasny, B.R., *Science 238*:1653 (1987); Miller, D.W., *et al.*, in *Genetic Engineering* (1986), Setlow, J.K., *et al.*, eds., *Plenum*, Vol. 8, pp. 277-297).

As discussed above, expression of Jagged in eukaryotic hosts requires the use of eukaryotic regulatory regions. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis. Preferred eukaryotic promoters include: the promoter of the mouse metallothionein I gene sequence (Hamer, D., *et al.*, *J. Mol. Appl. Gen. 1*:273-288 (1982)); the TK promoter of Herpes virus (McKnight, S., *Cell 31*:355-365 (1982)); the SV40 early promoter (Benoist, C., *et al.*, *Nature (London) 290*:304-310 (1981)); the yeast *gal4* gene sequence promoter (Johnston, S.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 79*:6971-6975 (1982); Silver, P.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 81*:5951-5955 (1984)).

As is widely known, translation of eukaryotic mRNA is initiated at the codon which encodes the first methionine. For this reason, it is preferable to ensure that the linkage between a eukaryotic promoter and a DNA sequence which encodes Jagged (or a functional derivative thereof) does not contain any intervening codons which are capable of encoding a methionine (*i.e.*, AUG). The presence of such codons results either in a formation of a fusion protein (if the AUG codon is in the same reading frame as the Jagged coding sequence) or a frame-shift mutation (if the AUG codon is not in the same reading frame as the Jagged coding sequence).

The Jagged coding sequence and an operably linked promoter may be introduced into a recipient prokaryotic or eukaryotic cell either as a non-replicating DNA (or RNA) molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule.

Since such molecules are incapable of autonomous replication, the expression of the Jagged may occur through the transient expression of the introduced sequence. Alternatively, permanent expression may occur through the integration of the introduced sequence into the host chromosome.

In one embodiment, a vector is employed which is capable of integrating the desired gene sequences into the host cell chromosome. Cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may provide for prototrophy to an auxotrophic host, biocide resistance, e.g., antibiotics, or heavy metals, such as copper, or the like. The selectable marker gene sequence can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of single chain binding protein mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama, H., *Molec. Cell. Biol. 3*:280 (1983).

In a preferred embodiment, the introduced sequence will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Preferred prokaryotic vectors include plasmids, such as those capable of replication in *E. coli* (such as, for example, pBR322, ColE1, pSC101, pACYC 184, πVX. Such plasmids are, for example, disclosed by Maniatis, T., *et al.* (In: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY (1982)). *Bacillus* plasmids include pC194, pC221, pT127, etc. Such plasmids are disclosed by Gryczan, T. (In: *The Molecular Biology of the Bacilli*, Academic Press, NY (1982), pp. 307-329). Suitable *Streptomyces* plasmids include pIJ101 (Kendall, K.J., *et al., J. Bacteriol. 169*:4177-4183 (1987)), and streptomyces bacteriophages such as φC31 (Chater, K.F., *et al.*, In: *Sixth*

International Symposium on Actinomycetales Biology, Akademiai Kaido, Budapest, Hungary (1986), pp. 45-54). Pseudomonas plasmids are reviewed by John, J.F., *et al.* (*Rev. Infect. Dis.* *8*:693-704 (1986)), and Izaki, K. (*Jpn. J. Bacteriol.* *33*:729-742 (1978)).

Preferred eukaryotic plasmids include BPV, vaccinia, SV40, 2-micron circle, etc., or their derivatives. Such plasmids are well known in the art (Botstein, D., *et al.*, *Miami Wntr. Symp.* *19*:265-274 (1982); Broach, J.R., In: *The Molecular Biology of the Yeast Saccharomyces*: Life Cycle and Inheritance, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 445-470 (1981); Broach, J.R., *Cell 28*:203-204 (1982); Bollon, D.P., *et al.*, *J. Clin. Hematol. Oncol.* *10*:39-48 (1980); Maniatis, T., In: *Cell Biology: A Comprehensive Treatise*, Vol. 3, Gene sequence Expression, Academic Press, NY, pp. 563-608 (1980)).

Once the vector or DNA sequence containing the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means: transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection, etc. After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of Jagged, or fragments thereof. This can take place in the transformed cells as such, or following the induction of these cells to differentiate (for example, by administration of bromodeoxyuracil to neuroblastoma cells or the like).

The Jagged proteins (or a functional derivatives thereof) of the present invention can be used in a variety of procedures and methods, such as for the generation of antibodies, for use in identifying pharmaceutical compositions, and for studying DNA/protein interaction.

The peptides of the present invention may also be administered to a mammal intravenously, intramuscularly, subcutaneously, enterally, topically or parenterally. When administering peptides by injection, the administration may be by continuous injections, or by single or multiple injections. The peptides are intended to be provided to a recipient mammal in a "pharmacologically or pharmaceutically acceptable form" in an amount sufficient to "therapeutically effective." A peptide is considered to be in "pharmaceutically or pharmacologically acceptable form" if its administration can be tolerated by a recipient patient. An amount is said to be "therapeutically effective" (an "effective amount") if the dosage, route

of administration, etc., of the agent are sufficient to affect a response to Jagged. Thus, the present peptides may be used to increase or enhance the effect of the Jagged protein.

In another embodiment of the present invention, methods for inhibiting, decreasing or preventing the activity of the Jagged peptide can be achieved by providing an agent capable of binding to the ligand (or a functional derivative thereof). Such agents include, but are not limited to: antisense Jagged, the antibodies to Jagged (anti-Jagged), and the secondary or anti-peptide peptides of the present invention. By decreasing the activity of Jagged, the effects which the expression of the peptide has on angiogenesis or restenosis can be modified.

In one example of the present invention, methods are presented for decreasing the expression of Jagged (or a functional derivative thereof) by means of an anti-sense strand of cDNA to disrupt the translation of the Jagged message. Specifically, a cell is modified using routine procedures such that it expresses an antisense message, a message which is complementary to the pseudogene message. By constitutively or inducibly expressing the antisense RNA, the translation of the Jagged mRNA can be regulated. Such antisense technology has been successfully applied to regulate the expression of poly(ADP-ribose) polymerase (*see*, Ding *et al.*, *J. Biol. Chem. 267* (1992)).

On the other hand, methods for stimulating, increasing or enhancing the activity of the Jagged peptide can be achieved by providing an agent capable of enhancing the binding capability or capacity of the ligand (or a functional derivative thereof), or by inhibiting or preventing a signal which would diminish or stop the expression of Jagged in the system. Such agents include, but are not limited to, the anti-antisense Jagged peptides of the present invention. By enhancing the activity of Jagged, the affect which the expression of the peptide has on angiogenesis or restenosis can also be modified.

In yet another embodiment, Jagged (or a functional derivative or variant thereof) can be used to produce antibodies or hybridomas. One skilled in the art will recognize that if an antibody is desired that will bind to Jagged, such a ligand would be generated as described above and used as an immunogen. The resulting antibodies are then screened for the ability to bind Jagged. Additionally, the antibody can be screened for its inability to bind Notch.

The antibodies utilized in the above methods can be monoclonal or polyclonal antibodies, as well fragments of these antibodies and humanized forms. Humanized forms of

the antibodies of the present invention may be generated using one of the procedures known in the art such as chimerization or CDR grafting.

In general, techniques for preparing monoclonal antibodies are well known in the art (Campbell, A.M., "*Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology*," Elsevier Science Publishers, Amsterdam, The Netherlands (1984); St. Groth *et al.*, *J. Immunol. Methods 35*:1-21 (1980). For example, in one embodiment an antibody capable of binding Jagged is generated by immunizing an animal with a synthetic polypeptide whose sequence is obtained from a region of the Jagged protein.

Any animal (mouse, rabbit, etc.) which is known to produce antibodies can be utilized to produce antibodies with the desired specificity, although because of the large size of the Jagged molecule, the rabbit is more preferred. Methods for immunization are well known in the art. Such methods include subcutaneous or interperitoneal injection of the polypeptide. One skilled in the art will recognize that the amount of polypeptide used for immunization will vary based on the animal which is immunized, the antigenicity of the polypeptide and the site of injection.

The polypeptide may be modified or administered in an adjuvant in order to increase the peptide antigenicity. Methods of increasing the antigenicity of a polypeptide are well known in the art. Such procedures include coupling the antigen with a heterologous protein (such as globulin or β-galactosidase) or through the inclusion of an adjuvant during immunization.

For monoclonal antibodies, spleen cells from the immunized animals are removed, fused with myeloma cells, such as SP2/0-Ag14 myeloma cells, and allowed to become monoclonal antibody producing hybridoma cells. A hybridoma is an immortalized cell line which is capable of secreting a specific monoclonal antibody.

Any one of a number of methods well known in the art can be used to identify the hybridoma cell which produces an antibody with the desired characteristics. These include screening the hybridomas with an ELISA assay, western blot analysis, or radioimmunoassay (Lutz *et al.*, *Exp. Cell Res. 175*:109-124 (1988)).

Hybridomas secreting the desired antibodies are cloned and the class and subclass are determined using procedures known in the art (Campbell, A.M., *Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers, Amsterdam, The Netherlands (1984)).

For polyclonal antibodies, antibody containing antisera is isolated from the immunized animal and is screened for the presence of antibodies with the desired specificity using one of the above-described procedures.

Conditions for incubating an antibody with a test sample vary. Incubating conditions depend on the format employed in the assay, the detection methods employed the nature of the test sample, and the type and nature of the antibody used in the assay. One skilled in the art will recognize that any one of the commonly available immunological assay formats (such as, radioimmunoassays, enzyme-linked immunosorbent assays, diffusion based Ouchterlony, or rocket immunofluorescent assays, or the like) can readily be adapted to employ the antibodies of the present invention. Examples of such assays can be found in Chard, T. "*An Introduction to Radioimmunoassay and Related Techniques*" Elsevier Science Publishers, Amsterdam, The Netherlands (1986); Bullock, G.R. *et al.*, "*Techniques in Immunocytochemistry,*" Academic Press, Orlando, FL Vol. 1 (1982), Vol. 2 (1983), Vol. 3 (1985); Tijssen, P., "*Practice and Theory of Enzyme Immunoassays: Laboratory Techniques in Biochemistry and Molecular Biology,*" Elsevier Science Publishers, Amsterdam, The Netherlands (1985).

The anti-Jagged antibody is also effective when immobilized on a solid support. Examples of such solid supports include, but are not limited to, plastics such as polycarbonate, complex carbohydrates such as agarose and sepharose, and acrylic resins, such as polyacrylamide and latex beads. Techniques for coupling antibodies to such solid supports are well known in the art (Weir, D.M. *et al.*, "*Handbook of Experimental Immunology*" 4th Ed., Blackwell Scientific Publications, Oxford, England, Chapter 10 (1986), Jacoby, W.D. *et al.*, *Meth. Enzym. 34* Academic Press, N.Y. (1974).

Additionally, one or more of the antibodies used in the above described methods can be detectably labelled prior to use. Antibodies can be detectably labelled through the use of radioisotopes, affinity labels (such as, biotin, avidin, etc.), enzymatic labels (such as, horse radish peroxidase, alkaline phosphatase, etc.) fluorescent labels (such as, FITC or rhodamine, etc.), paramagnetic atoms, etc. Procedures for accomplishing such labelling are well-known in the art, for example see Sternberger, L.A. *et al.*, *J. Histochem. Cytochem. 18*:315 (1970), Bayer, E.A. *et al.*, *Meth. Enzym. 62*:308 (1979), Engval, E. *et al.*, *Immunol. 109*:129 (1972), Goding, J.W. *J. Immunol. Meth. 13*:215 (1976). The labeled antibodies of the present invention

can be used for *in vitro*, *in vivo*, and *in situ* assays to identify cells or tissues which express a specific protein or ligand.

In an embodiment of the above methods, the antibodies are labeled, such that a signal is produced when the antibody(s) bind to the same molecule. One such system is described in U.S. Patent No. 4,663,278.

The antibodies or antisense peptides of the present invention may be administered to a mammal intravenously, intramuscularly, subcutaneously, enterally, topically or parenterally. When administering antibodies or peptides by injection, the administration may be by continuous injections, or by single or multiple injections.

The antibodies or antisense peptides of the present invention are intended to be provided to a recipient mammal in a "pharmaceutically acceptable form" in an amount sufficient to be "therapeutically effective" or an "effective amount". As above, an amount is said to be therapeutically effective (an effective amount), if the dosage, route of administration, etc. of the agent are sufficient to affect the response to Jagged. Thus, the present antibodies may either stimulate or enhance the effect of the Jagged protein, or they may inhibit or prevent the effect of the Jagged protein. Or, secondary antibody(s) may be designed to affect the response to the Jagged antibody(s) per se, *i.e.*, an anti-antibody to Jagged. In the alternative, either an antibody or an anti-antibody may be designed to affect only the anti-sense strand of the ligand.

One skilled in the art can readily adapt currently available procedures to generate secondary antibody peptides capable of binding to a specific peptide sequence in order to generate rationally designed antipeptide peptides, for example see Hurby et al., Application of Synthetic Peptides: Antisense Peptides", In *Synthetic Peptides, A User's Guide*, W.H. Freeman, NY, pp. 289-307 (1992), and Kaspczak *et al.*, *Biochemistry 28*:9230-8 (1989). As used herein, an agent is said to be "rationally selected or designed" when the agent is chosen based on the configuration of the Jagged peptide.

Anti-peptide peptides can be generated in one of two fashions. First, the anti-peptide peptides can be generated by replacing the basic amino acid residues found in the pseudogene peptide sequence with acidic residues, while maintaining hydrophobic and uncharged polar groups. For example, lysine, arginine, and/or histidine residues are replaced with aspartic acid or glutamic acid and glutamic acid residues are replaced by lysine, arginine or histidine. Alternatively, the anti-peptide peptides of the present invention can be generated by

synthesizing and expressing a peptide encoded by the antisense strand of the DNA which encodes the pseudogene peptide. Peptides produced in this fashion are, in general, similar to those described above since codons complementary to those coding for basic residues generally code for acidic residues.

To detect secondary antibodies, or in the alternative, the labelled primary antibody, labeling reagents may include, *e.g.*, chromophobic, enzymatic, or antibody binding reagents which are capable of reacting with the labeled antibody. One skilled in the art will readily recognize that the disclosed antibodies of the present invention can readily be incorporated into one of the established kit formats which are well known in the art.

An antibody is said to be in "pharmaceutically or pharmacologically acceptable form" if its administration can be tolerated by a recipient patient. The antibodies of the present invention can be formulated according to known methods of preparing pharmaceutically useful compositions, whereby these materials, or their functional derivatives, are combined with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in Remington's Pharmaceutical Sciences, 1980).

In order to form a pharmaceutically acceptable composition which is suitable for effective administration, such compositions will contain an effective amount of an antibody of the present invention together with a suitable amount of carrier. Such carriers include, but are not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and a combination thereof. The carrier composition may be sterile. The formulation should suit the mode of administration. In addition to carriers, the antibodies of the present invention may be supplied in humanized form.

Humanized antibodies may be produced, for example by replacing an immunogenic portion of an antibody with a corresponding, but non-immunogenic portion (*i.e.*, chimeric antibodies) (Robinson *et al.*, International Patent Publication PCT/US86/02269; Akira *et al.*, European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al.*, European Patent Application 173,494; Neuberger, M.S. *et al.*, PCT Application WO 86/01533; Cabilly *et al.*, European Patent Application 125,023; Better *et al.*, *Science 240*:1041-1043 (1988); Liu *et al.*, *Proc. Natl. Acad. Sci. USA 84*:3439-3443 (1987); Liu *et al.*, *J. Immunol. 139*:3521-3526 (1987); Sun *et al.*, *Proc. Natl. Acad. Sci. USA*

84:214-218 (1987); Nishimura *et al., Canc. Res. 47*:999-1005 (1987); Wood *et al., Nature 314*:446-449 (1985)); Shaw *et al., J. Natl.Cancer Inst. 80*:1553-1559 (1988).

The compositions of the present invention can also include minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation or powder. The composition can be formulated as a suppository with traditional binders and carriers such as triglycerides. Oral formulations can include standard carriers such as pharmaceutically acceptable mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

In a preferred embodiment of the present invention, the compositions are formulated in accordance with routine procedures for intravenous administration to a subject. Typically, such compositions are carried in a sterile isotonic aqueous buffer. As needed, a composition may include a solubilizing agent and a local anesthetic. Generally, the components are supplied separately or as a mixture in unit dosage form, such as a dry lyophilized powder in a sealed container with an indication of active agent. Where the composition is administered by infusion, it may be provided with an infusion container with a sterile pharmaceutically acceptable carrier. When the composition is administered by injection, an ampoule of sterile water or buffer may be included to be mixed prior to injection.

The therapeutic compositions may also be formulated in salt form. Pharmaceutically acceptable salts include those formed with free amino groups, such as those derived from hydrochloric, phosphoric, acetic, oxalic and tartaric acids, or formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides. isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The dosage of the administered agent will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of the antibody which is in the range of from about 1 pg/kg to 10 mg/kg (body weight of patient), although a lower or higher dosage may be administered. Suitable ranges for intravenous administration is typically about 20-500 μg of active compound per kilogram body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* and *in vivo* animal model test systems.

Since highly purified proteins are now available, X-ray crystallography and NMR-imaging techniques can be used to identify the structure of the ligand binding site. Utilizing such information, computer modeling systems are now available that allows one to "rationally design" an agent capable of binding to a defined structure (Hodgson, *Biotechnology 8*:1245-1247 (1990)), Hodgson, *Biotechnology 9*:609-613 (1991)). As used herein, an agent is said to be "rationally designed" if it is selected based on a computer model of the ligand or Notch binding site, or in the alternative, of the ligand binding site on Jagged if activation of the Notch binding site is found to act as an on/off switch affecting the continued expression of Jagged.

In another embodiment of the present invention, methods are provided for modulating the translation of RNA encoding Jagged protein in the cell. Specifically, said method comprises introducing into a cell a DNA sequence which is capable of transcribing RNA which is complimentary to the RNA encoding the Jagged protein. By introducing such a DNA sequence into a cell, antisense RNA will be produced which will hybridize and block the translation of the Jagged protein. Antisense cloning has been described by Rosenberg *et al.*, *Nature 313*:703-706 (1985), Preiss *et al.*, *Nature 313*:27-32 (1985), Melton, *Proc. Natl. Acad. Sci. USA 82*:144-148 (1985) and Kim *et al.*, *Cell 42*:129-138 (1985).

Transcription of the introduced DNA will result in multiple copies of antisense RNA which will be complimentary to the Jagged. By controlling the level of transcription of antisense RNA, and the tissue specificity of expression, one skilled in the art can regulate the level of translation of Jagged protein in specific cells within a patient.

In one aspect of the above-described invention, DNA response elements (RE) can be identified which are capable of either stimulating or inhibiting the binding of Jagged. In this manner, assays may be performed to determine binding agents by using any length of DNA so long as it contains at least one RE sequence. In another embodiment, the above such assays are performed in the absence of a RE. In this fashion, agents can be identified which bind to or affect the binding capacity of Jagged independently of DNA binding. Moreover, the above assay can be modified so that it is capable of identifying agents which activate transcription of DNA sequences controlled by a RE.

In the present invention, a cell or organism is altered using routine methods such that it expresses Jagged, or a functional derivative thereof. Moreover, the cell or organism may be further altered to contain a RE operably linked to a reporter sequence, such as luciferase, beta

galactosidase, or chloramphenicol acyltransferase. Agents are then incubated with the cell or organism and the expression of the reporter sequence is assayed.

In an alternative usage, nuclear and/or cytosolic extracts from the altered cell containing Jagged or a functional derivative thereof are mixed with an expression module containing an RE operably linked to a reporter sequence. The extract/expression module is incubated with an agent and the expression of the reporter sequence is assayed.

In another embodiment of the present invention, kits are provided which contain the necessary reagents to carry out the previously described methods and assays.

The present invention further provides methods of regulating gene expression in a cell. For example, a cell can be altered such that it contains a DNA sequence operably linked to an RE. Additionally, the cell can be altered to control the expression of Jagged. permitting one skilled in the art to generate a cell which expresses a given sequence in response to a particular agent.

The subjects treated in accordance with the present invention include any vertebrate organism; more preferably any mammal; most preferably a human. The only limiting factor is that the organism endogenously produces Notch and/or the toporythmic genes which modulate binding to Notch.

By providing methods of affecting angiogenesis by modulating the Notch-Jagged signal pathway, the present invention provides methods and compositions which affect a number of physiologic and pathologic conditions, including placental development, wound healing, rheumatoid arthritis, diabetic retinopathy and solid tumor growth and metastasis and motor neuron disorders. The referenced wound healing includes healing of any injury or lesion in the skin, tissue, vasculature, or nervous system of the subject, and includes cell migration and differentiation of cells comprising the mesoderm, endoderm, ectoderm and/or neuroderm. The wound or injury can be the result of surgery, trauma, and/or disease or condition. Such disease and/or conditions include ischemic lesions resulting from a lack of oxygen to the cell or tissue, *e.g.*, cerebral or cardiac infarction or ischemia, malignant lesions, infectious lesions, *e.g.*, abscess, degenerative lesions, lesions related to nutritional disorders, neurological lesions associated with systemic diseases, *e.g.*, diabetic neuropathy and retinopathy, systemic lupus erythematosus, carcinoma or sarcoidosis, and lesions caused by toxins, *e.g.*, alcohol, lead, etc. Motor neuron disorders may include, *e.g.*, amylotrophic lateral sclerosis, progressive spinal

muscular atrophy, progressive bulbar palsy, primary lateral sclerosis, infantile and juvenile muscular atrophy, progressive bulbar paralysis of childhood (Fazio-Londe syndrome), poliomyelitis and the post polio syndrome, and hereditary Motorsensory Neuropathy (Charcot-Marie-Tooth disease).

All essential publications mentioned herein are hereby incorporated by reference.

In order that those skilled in the art can more fully understand this invention, the following examples are set forth. These examples are included solely for the purpose of illustration, and should not be considered as expressing limitations unless so set forth in the appended claims.

EXAMPLES

In the following examples and protocols, restriction enzymes, ligase, labels, and all commercially available reagents were utilized in accordance with the manufacturer's recommendations. The cell and molecular methods utilized in this application are established in the art and will not be described in detail. However, standard methods and techniques for cloning, isolation, purification, labeling, and the like, as well as the preparation of standard reagents were performed essentially in accordance with *Molecular Cloning: A Laboratory Manual, second edition*, edited by Sambrook, Fritsch & Maniatis, Cold Spring Harbor Laboratory, 1989, and the revised third edition thereof, or as set forth in the literature references cited and incorporated herein. Methodologic details may be readily derived from the cited publications.

Example 1

Isolation of Human Endothelial Cell cDNA Induced by Exposure to Fibrin

Endothelial cells plated on fibrin organize into three dimensional tubular structures *in vitro* (Olander *et al.*, *J. Cell. Physiol.* *125*:1-9 (1985)), and this organizational behavior requires transcriptional responses (Zimrin *et al.*, 1995). Using a modification of the differential display, cDNA clones were obtained that were differentially expressed by HUVECs in response to fibrin. Briefly, total RNA was isolated from HUVEC plated on fibrin in the presence of crude FGF-1 at 0, 2, 5 and 24 hours and subjected to the modified differential mRNA display. One of the clones (D9) isolated from HUVEC populations exposed to fibrin, which was found to

have increased at the 2 hour time-point, was cloned and sequenced. A search of the GenBank database in 1994 demonstrated that the D9 sequence was novel.

The D9 clone (SEQ ID NO:2) was used as a probe to screen a lambda cDNA library prepared from mRNA obtained from HUVECs exposed to fibrin gels for 1, 3 and 5 hours. Ten isolates were recovered that contained the D9 sequence, two of which appeared, by restriction enzyme analysis, to be spliced variants of the remaining eight. Sequence analysis of the clones revealed that they overlapped to form a contiguous sequence of 5454 base pairs (bp) in length, set forth as SEQ ID NO:1.

Example 2

Analysis of the Sequence of HUVEC Clone D9 Demonstrates Homology with the Rat Jagged Gene

A second search of the Genebank database in 1995 revealed that the D9 clone was very homologous to the cDNA sequence coding for the rat Jagged gene (Lindsell *et al.*, 1995), a ligand for the Notch receptor. Computer analysis revealed an 87% identity at the nucleotide level and a 95 % identity at the amino acid level. The Jagged protein contains a putative signal sequence, a DSL domain which describes a consensus region present in other Notch ligands (Delta, Serrate, Lag-2 and Apx-1), an EGF-like repeat domain containing 16 EGF repeats, a cys-rich domain, a transmembrane domain, and a 125 residue cytosol domain. This structure is represented in Figure 2. Thus, it was determined that clone D9 represents the human homolog of the rat Jagged cDNA.

Two additional Jagged clones were also obtained each containing identical deletions. The first was 89 bp in length, and was located in the middle of the cys-rich region. The second occurred 366 bp downstream from the first region, and was 1307 bp in length. The first deletion predicts a frame-shift in the translation product, resulting in a unique 15 amino acid sequence followed by a premature termination of the protein, effectively deleting the transmembrane and cytosol domain from the Jagged structure.

Example 3

Human Endothelial Cell Populations Express Both Jagged and Notch Transcripts

To ascertain that both the human Jagged gene and its putative receptor, Notch, were expressed in the HUVEC population, oligonucleotide primers were designed based upon the published sequence for the human Tan-1 transcript (Notch-1) and the human Notch group protein transcript (Notch-2), as well as for the human Jagged transcript.

Total RNA was obtained using standard protocols. The differential display was performed as previously described by Folkman and Haudenschild, *Nature 288*:551-556 (1980). Briefly, 1 μg of total RNA was reverse transcribed with 200U M-MLV reverse transcriptase (BRL) in the presence of 2 μM of the 3' primer (5'-GCGCAAGCT$_{12}$CG-3') and 100 μM dNTP for 70 minutes at 37°C. The cDNA was amplified in the presence of (^{32}P) dATP (Amersham) using the same 3' primer and a 5' primer with the sequence 5'-GAGACCGTGAAGATACTT-3' and the following parameters: 94°C 45 seconds, 41°C 1 minute, 72°C 1 minute for 4 cycles, followed by 94°C 45 seconds, 60°C 1 minute, 72°C 1 minute for 18 cycles. The resulting cDNA species were separated using polyacrylamide gel electrophoresis, the gel was dried and exposed to radiographic film, and the band of interest was cut out of the gel and eluted.

The cDNA was amplified using the same primers and cloned into a TA vector (Invitrogen). The clone was used to screen a cDNA library made in the ZAP Express vector (Stratagene) using RNA isolated from HUVEC plated on fibrin in the presence of crude FGF-1 for 1, 3, 5, 8 and 24 hours to analyze the steady-state levels of the transcripts for Jagged, Notch 1, Notch 2, and GAPDH. *See*, Garfinkel *et al.*, submitted *J. Cell Biol.* 1996. The overlapping cDNA clones obtained were sequenced using an ABI DNA synthesizer and assembled with the DNASTAR program. RT-PCR analysis was performed as described using the following primers:

jagged sense 5'-CCGACTGCAGAATAAACATC-3;
jagged antisense 5'-TTGGATCTGGTTCAGCTGCT-3';
notch 1 sense 5'-TTCAGTGACGGCCACTGTGA-3';
notch 1 antisense 5'-CACGTACATGAAGTGCAGCT-3';
notch 2 sense 5'-TGAGTAGGCTCCATCCAGTC-3';
notch 2 antisense 5'-TGGTGTCAGGTAGGGATGCT-3';

GAPDH sense 5'-CCACCCATGGCAAATTC-CATGGCA-3';
GAPDH antisense 5'-TCTAGACGGCAGGTCAGGTCCACC-3'.

As shown in Figure 5, the steady state levels of the Notch-1 and Notch-2 transcripts were not altered in HUVEC populations exposed to fibrin. In contrast, however, the HUVEC Jagged transcript was induced after three hours exposure to fibrin after which time the steady state levels of the Jagged transcript decreased (Figure 5).

Example 4

The Role of Jagged as a Mediator of Microvascular Sprout Formation In Vitro

Because (i) Delta/Serrate signaling through Notch is involved in the determination of cell fate in invertebrates (Fortini and Artavanis-Tsakonas, 1993), (ii) Jagged signaling through Notch attenuates the terminal differentiation of myoblasts to myotubes *in vitro* (Lindsell *et al.*, 1995), (iii) the endothelial cell presents a non-terminal differentiated phenotype *in vitro* (Figure 1), and (iv) the Jagged transcript was identified as an endothelial cell differentiation-induced gene, it was important to determine whether Jagged-Notch signaling in the endothelial cell was involved in the early phase of the differentiation pathway. It is well known that endothelial cell sprout formation is an early event in the microvasculature during angiogenesis (Montesano *et al.*, 1985); and endothelial cell sprout formation assays are described in the art (Montesano *et al., Proc. Natl. Acad. Sci. USA 83*:7297-7301 (1986)). However, to assess the role of Jagged-Notch signaling in this system, an antisense (γ) oligonucleotide was needed, based on the Jagged sequence to repress the translation of the Jagged transcript.

The γ-Jagged oligomer contained the Kozak sequence, the ATG translation start site and extended three codons into the open-reading frame. Similar γ-oligomers have proven useful in a wide variety of cellular systems to repress the translation of specific transcripts, including the human endothelial cell (Maier *et al.*, 1990b; Garfinkel *et al., J. Biol. Chem. 267*:24375-24378 (1992)). The controls for the γ-Jagged oligomer included the sense counterpart, a 3'-antisense oligomer and a mutated 5' antisense oligomer.

Although the complete DNA sequence of the bovine Jagged transcript had not yet been fully defined, a high degree of homology at the 5' end was predicted between the bovine and the human Jagged nucleotide sequence, in view of the fact that the human and rat Jagged polypeptides are 95 % identical.

Bovine microvascular endothelial cells (BMEC) were plated onto a collagen gel, grown to confluence in the presence or absence of varied concentrations of the γ-Jagged oligomer. FGF-2 (10 ng/ml) was added at confluence (Montesano *et al.*, 1986), and the length of microvessels (sprouts formed as a result of cellular invasion into the collagen gel) was measured (Pepper *et al.*, *Biochem. Biophys. Res. Comm. 189*:824-831 (1992)). As shown in Figure 6, exposure to the γ-Jagged oligomer resulted in an increase BMEC sprout length in a concentration dependent manner above the level achieved by FGF-2. In contrast, the three control oligomers, a Jagged sense oligonucleotide, a 3' antisense Jagged oligomer, and a mutated 5' antisense Jagged oligomer did not affect the ability of FGF-2 to induce sprout formation in this assay (Figure 6).

Prior to this experiment, with the possible exception of vascular endothelial cell growth factor (VEGF), no other growth factor/cytokine signal has been disclosed as able to potentiate the ability of FGF to modify BMEC sprout length. This result would not have been previously anticipated since the Jagged gene had been previously identified as a HUVEC-derived differentiation-induced transcript.

Example 5

The Disparate Effect of the Antisense (γ)-Jagged Oligomer on Small and Large Vessel Endothelial Cell Migration

Based upon the surprising effect of the γ-Jagged oligomers on the potentiation of FGF-2-induced BMBC sprout formation (Example 4), a simple assay was designed to assess the influence of the γ-Jagged oligomer on BMEC migration, specifically to confirm that interrupting the Jagged-Notch signaling pathway would attenuate the ability of FGF to increase sprout length. Utilizing essentially the system of Sato and Rifkin (1988, *supra*), bovine microvascular endothelial cells (BMEC) were plated on a fibronectin matrix, and grown to confluence in the absence and presence of varied amounts of the γ-Jagged oligomer.

Briefly, 4 x 10^5 BMEC and BAEC were grown to confluence in serum-containing media containing 0, 1.25, 2.5, 5 and 6.25 uM jagged antisense oligonucleotide. The monolayers were wounded by scraping them with a razor blade and cellular debris was removed by washing the plates twice with phosphate buffered saline. The cells were incubated for a further 22 hours at 37°C to confluence, then fixed in 25% acetic acid, 75% methanol and stained with hematoxylin (Sigma). The number of cells migrating from the wound origin were counted to determine the ability of the BMEC population to migrate into the denuded area. The count was made using a light microscope with a grid at 100x magnification. The data represent a mean of multiple experiments done in duplicate, with five microscopic fields counted for each point.

As shown in Figure 7A, the presence of the γ-Jagged oligomer resulted in an increase in the number of cells migrating into the denuded area with an approximate 80% increase mediated by 5 μM γ-Jagged oligomer. These data (Figure 7A) agree with the BMEC data obtained from the sprout assay in which 2 μM γ-Jagged oligomer yielded an approximate 100% increase in BMEC sprout length (Figure 6). Thus, it was shown that an interruption in the Jagged-Notch signaling pathway resulted in an *increase* in BMEC migration, a major immediate-early component of sprout formation *in vitro*.

Consequently, an apparent discrepancy was noted between the results of the experiments showing (i) the isolation of the Jagged transcript from a HUVEC population preparing to migrate into a fibrin gel, and (ii) the enhancement of the BMEC by the presumed interruption of the Jagged signal. Noting that the HUVEC are obtained from a macro-vessel, and BMEC are from micro-vessels, the distinction was apparently directly related to the nature of the source of the endothelial cells.

To ascertain that the difference was based upon the type of the endothelial cell (macro- versus micro-vasculature), and not due to variations in the extracellular matrix or the function of growth factors/cytokines in the particular system, an experiment was designed in which the endothelial cells were obtained from the same species, but exclusively from a macrovascular source - bovine aorta endothelial cells (BAEC). BAEC were introduced onto a fibronectin matrix, grown to confluence in the absence and presence of various amounts of the γ-Jagged oligomer, and their migration assessed in a manner identical to that used to assess BMEC migration. As shown in Figure 7B, there was a concentration-dependent *decrease* in the

migration of the BAEC population in response to the γ-Jagged oligomer with an approximate 50% reduction in BAEC migration at 5 μM γ-Jagged oligomer.

When viewed together, these results indicated Jagged-Notch signaling as an *anti*-migratory event in the endothelium comprising the microvasculature, but as a *pro*-migratory event in the endothelium of large vessels. These experiments demonstrated for the first time that there apparently exists a major phenotype difference between small and large vessel endothelial cells in response to a ligand-receptor signaling pathway in the endothelial cell which is modulated during the migratory phase of angiogenesis.

Example 6

Further Characterization of the Disparate Effects Mediated by Jagged-Induced Signaling *In Vitro* Using Human Endothelial Cells

To better understand the mechanism utilized by human endothelial cells to regulate angiogenesis in man, it is important to study the effect of the γ-Jagged oligomer on cell migration using human microvascular endothelial cells and human endothelial cells from large vessels. Although it would be preferable to obtain stable human endothelial cell γ-Jagged transfectants/transductants using conventional gene transfer methods, none have proven useful with regard to human diploid endothelial cells *in vitro*. Therefore, the γ-Jagged oligomer strategy is employed as a means to modify the translational efficiency of the human Jagged transcript.

Initially, however, two methods are used to confirm that the γ-Jagged oligomer is able to reduce the efficiency of Jagged translation. Each utilizes rabbit anti-Jagged antibodies being prepared against individual synthetic peptides derived from the extracellular DSL domain, the extracellular cys-poor domain (NH_2-terminal to the transmembrane domain) and the intracellular domain of the predicted Jagged protein sequence. Immunologic methods parallel those previously used for the production and purification of antibodies against synthetic peptides derived from sequence analysis of the FGF-1 receptor (Prudovsky *et al.*, *J. Biol. Chem.* *269*:31720-31724 (1994)), cortactin (Zhan *et al.*, 1994) and FGF-1 (Imamura *et al.*, *Science* *249*:1567-1570 (1990)) translation products. Synthetic peptides are prepared as multiple antigen peptides (MAP) using fmoc MAP resins from Applied Biosystems. Likewise,

Notch-1 antibodies are also prepared using sequence from the extracellular LNG domain and intracellular ankyrin repeat domain for MAP synthesis.

The first method utilizes hybrid selection, using an immobilized Jagged oligomer to capture the Jagged transcript from HUVEC populations, followed by (^{35}S)-met/cys translation of the Jagged transcript in the rabbit reticulocyte system in the absence and presence of varied amounts of the γ-Jagged oligomer. Immunoprecipitation of the Jagged protein followed by SDS-PAGE autoradiography establishes the ability of the γ-Jagged oligomer to repress Jagged translation *in vitro*.

The second method utilizes HUVEC populations metabolically labeled with (^{35}S)-met/cys for Jagged immunoprecipitation from cells exposed to fibrin for 0, 1, 2 and 3 hours. Immunoprecipitation of the Jagged protein from the fibrin-induced HUVEC population followed by SDS-PAGE autoradiography permits a comparative assessment of whether pretreatment of the cells with the γ-Jagged oligomer represses the level of the Jagged protein as a cell-associated polypeptide. The success of these strategies is based upon the fact that the Jagged protein sequence is rich in cys residues, and as a result is metabolically labeled to a high specific activity. Likewise, an accurate molecular weight is assigned to the Jagged protein since competition with synthetic peptide, pre-immune serum, as well as denatured γ-Jagged antiserum, are used as controls to define the specificity of band assignment. Since the predicted Jagged translation product contains 1197 amino acids, the molecular weight is in the 135 to 145 kDa range.

The disparate migratory behavior of the BMEC and BAEC populations is confirmed using stable γ-Jagged transfectants. Since bovine cells are more amenable than HUVEC populations to gene transfer methods, the pMEXneo vector (Martin-Zanca *et al.*, *Mol. Cell. Biol.* 9:24-33 (1989)) is used to select for stable BMEC and BAEC γ-Jagged transfectants as previously described (Zhan *et al.*, 1992). Stable clones are obtained using G418 resistance to quantify the migratory potential of these cells relative to insert-less vector control transfectants. The wound-induced migration assay (Example 6; Figure 7) is useful to demonstrate that the serum-induced migration potential of the BMEC γ-Jagged transfectants is increased, and the serum-induced migration potential of the BAEC γ-Jagged transfectants is decreased.

Use of these transfectants permits a more rigorous quantification of the disparate modulation of migratory potential between small and large vessel endothelial cells using the

conventional Boyden chamber assay previously used to establish the chemotactic activity of FGF-1 (Terranova *et al., J. Cell Biol. 101*:2330-2334 (1985)). In addition, this approach also confirms the assessment of the ability of the BAEC γ-Jagged and insert-less vector control transfectants to respond to the FGF prototypes as inducers of sprout formation *in vitro* (Figure 6). Lastly, this strategy permits an assessment of the migratory responsiveness of additional bovine endothelial cells obtained from alternative anatomic sites, including the portal vein, saphenous artery and vein, and adipose-derived microvascular endothelial cells. The ability of these cells to induce steady-state levels of Jagged and Notch receptor transcripts in response to fibrin is also evalulated by RT-PCR analysis as in Example 3 (Figure 5).

A nuclear run-on analysis of BMEC and BAEC populations, as well as a kinetic analysis of the presence of the Jagged transcript in actinomycin D- and cycloheximide-treated cells in response to fibrin, is employed to determine whether the induction of the Jagged transcript is due to a transcriptional regulatory event and whether Jagged transcript stability is involved in the fibrin response. This analysis is analogous to a previous study on the post-transcriptional regulation of IL-1α in HUVEC populations by Garfinkel *et al., Proc. Natl. Acad. Sci. USA 91*:1559-1563 (1994). Nuclear run-on analysis is performed by incubating nuclei obtained from either BMEC or BAEC populations exposed to fibrin for 0, 1, 3 and 6 hours with 100 μCi of (^{32}P)-UTP for 30 minutes. This is followed by the isolation of nascent RNA transcripts, and slot blot analysis using 5 μg of the linearized, denatured and immobilized Jagged cDNA and hybridization at high stringency with the labeled RNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is used as a positive control, and densitometric values are normalized to the GAPDH signal. Although the level of the Jagged transcript may be difficult to predict, a Jagged signal should be visible. Testing the γ-Jagged oligomer at varied levels permits a determination of the ability, if any, of the γ-Jagged oligomer to access the transcriptional machinery in this system.

The analysis of the affect of the novel protein on human endothelial cells effectively employs the HUVEC population as a model, in comparison with HU artery (A) EC and human cells obtained from other anatomic sites, including, *e.g.*, human adipose-derived microvascular endothelial cells (HMEC), human dermis-derived capillary endothelial cells (HCEC) and human saphenous vein (HSVEC) and artery (HSAEC), available from commercial and academic sources. The addition of the γ-Jagged oligomer to these populations of human endothelial cells

will be similar to that described in the protocols involving bovine endothelial cell populations. Thus, the ability of the γ-Jagged oligomer to modulate sprout formation of human capillary, artery and vein endothelial cells is assessed using the collagen invasion assay described in Figure 6, and the migration wound assay described in Figure 7 supplemented with a Boyden chamber chemotaxis assay as previously described (Terranova *et al.*, 1985). The resulting data, similar to those obtained with the bovine endothelial cell populations, confirms the above-described conclusion (Examples 4 and 5) that reduction in the translational efficiency of the Jagged transcript (i) increases human microvascular endothelial cell sprout formation and migratory/chemotactic potential and (ii) reduces these activities in the human endothelial cell populations derived from arteries and veins.

To determine in those endothelial cell populations that are induced by the γ-Jagged oligomer to decrease tube formation, it is useful to evaluate whether there is a modification of the steady state transcript levels of the immediate-early endothelial differentiation genes (edg genes). This establishes whether the effect of the γ-Jagged oligomer occurs during immediate-early or mid-to-late phase of the endothelial cell differentiation pathway and supplements the qualitative data with respect to the modification of lumen formation *in vitro*. While the end point for this assay will be a qualitative assessment of lumen formation as previously described (Jaye *et al.*, 1985), cells will be harvested as described in Example 3 (Figure 5) for Northern blot analysis of the presence or absence of the edg genes, such as the G-protein-coupled orphan receptor, edg-1 (Hla and Maciag, 1990b), the transcription factor, edg-2 (Hla *et al.*, *Biochim. Biophys. Acta 1260*:227-229 (1995)), cyclooxygenase-2 (cox-2) (Hla and Neilson, *Proc. Natl. Acad. Sci. USA 89*:7384-7388 (1992)), and tissue collagenase, among others (Hla and Maciag, 1990a).

Because the data indicate that the γ-Jagged oligomer accelerates capillary endothelial cell migration and sprout formation *in vitro*, the addition of the Jagged protein to these systems will have the opposite effect - inhibiting capillary endothelial cell migration and sprout formation and promoting large vessel-derived endothelial cell migration *in vitro*. However, two approaches may be used to evaluate this premise. The first involves the expression and purification of the Jagged polypeptide as a recombinant protein, and the second involves the expression of an extracellular and soluble Jagged construct. Although the predicted Jagged sequence does not contain any recognizable post-translational modification motif in the

extracellular domain of the protein, such as N-glycosylation, it is possible that a subtle modification of the Jagged protein will affect the activity of Jagged as a Notch ligand.

Using the recombinant Jagged protein, it is possible to assess its ability to signal through the Notch-1 receptor using a rat myoblast system. Since it has been demonstrated that the rat myoblast cell line, C2C12, transfected with the Notch-1 cDNA will not form myotubes when co-cultured with a lethally irradiated population of murine fibroblast transfected with the rat Jagged cDNA (Lindsell *et al.*, 1995), it is assumed that the parental C2C12 is a Notch-1-deficient cell line. Therefore, the C2C12 cell represents a model cell type to assess the biological function of recombinant Jagged.

The C2C12 cell Notch-1 transfectants, but not C2C12 insert-less vector transfectants, presumably are unable to form myotubes if the recombinant Jagged protein is functional as a ligand. Thus, this system also permits an assessment of the value of Notch-2 as a Jagged receptor.

C2C12 cells are transfected with the full length rat Notch-1 and Notch-2 cDNA containing tandem copies of the influenza virus hemagglutinin (HA) epitope and stable transfectants obtained as described (Zhan *et al.*, 1992). The expression of the Notch-1 and Notch-2 receptor transcripts is monitored by RT-PCR and Northern blot analysis and the protein levels assessed by immunoprecipitation/Western blot analysis of the HA epitope. The addition of the recombinant Jagged ligand (1 ng to 10 μg titration) permits the Notch-1 and Notch-2 C2C12 cell transfectants to repress myotube formation, as assessed by morphologic criteria as well as by the repression of the steady-state levels of the myogenin transcript. These data also define the specific activity of the recombinant Jagged protein for stability studies (temperature, pH, ionic strength as a function of time). An appropriate positive control for these experiments is a population of lethally-irradiated NIH 3T3 cells transfected with the full-length Jagged cDNA to the Notch-1 and Notch-2 C2C12 cell transfectants, insuring the attenuation of myotube formation.

After the specific activity of the soluble Jagged protein is established, it will be possible to assess the ability of the Jagged ligand in a concentration dependent matter to inhibit microvessel endothelial cell migration, chemotaxis and sprout formation *in vitro*, as in Figures 5 and 6. Effective levels of Jagged protein, similar to those previously functional in the C2C12 cell Notch-1 transfectants, are expected to also be functional in the human and bovine

microvascular endothelial cell systems. A comparable evaluation involves a determination of the function of the Jagged protein as an inducer of large vessel-derived human and bovine endothelial cell migration, chemotaxis, and sprout formation. A concentration-dependent response is indicated. As described above, the co-culture of the large and small vessel-derived endothelial cells with lethally irradiated NIH 3T3 cell Jagged transfectants and insert-less vector transfectants provides a suitable control to demonstrate the disparate role of Jagged-Notch signaling in the regulation of endothelial cell migration.

Example 7

The Relevance of Jagged-Induced Signaling *In Vitro* to Angiogenesis *In Vivo*

Because Jagged was cloned as a fibrin-responsive gene *in vitro*, an *in vivo* angiogenic system is needed which closely mimics the *in vitro* system. Traditional angiogenesis assays, such as the chicken chonoallantoic membrane (CAM) (Scher *et al.*, *Cell 8*:373-382 (1976)) assay or the rabbit cornea assay (Folkman *et al.*, *Science 221*:719-725 (1983)), are useful for an end-point analysis, and are readily available in the art. However, the complexity of the many individual steps in the angiogenic cascade (Figure 1), and their control by gene regulation, demands a novel *in vivo* approach that addresses this complexity more specifically.

Plating HUVEC on fibrin has been selected to meet the need for such an *in vivo* system. It has proven to mimic *in vivo*, in a reproducible fashion, the *in vitro* system we used initially to induce and isolate the human Jagged cDNA. The *in vivo* system involves the subtotal occlusion of a large vessel, such as a carotid or iliac artery with a thrombus, producing an intimal injury. This is typically followed within two days, by migration of endothelial cells into the three-dimensional platelet/fibrin scaffold tube formation. After approximately 4 weeks the system characteristically displays tube perfusion, recruitment of pericytes, and selection of preferred channels with downsizing of minor vessels. Together with the vessels, stromal cells appear as well, contributing to the unique extracellular matrix of this tissue, and making this natural, *in vivo* system (involving revascularization of an experimental thrombus) ideal for demonstrating the role of Jagged and its receptor(s) in two of the early steps of angiogenesis.

Endothelial migration and tube formation can be separated in time (at 2, 4, 6, 8 days after thrombosis), as well as in space. The migrating cells are primarily located in the central

region of the thrombus, whereas the peripheral cells have already formed tubes, as indicated by the appearance of junctions and, almost concomitantly, the arrival of circulating red blood cells.

The antibodies developed for use in this experimental system were designed for use with known immunoperoxidase or immunofluorescence techniques to localize endogenous Jagged and Notch (Nabel et al., 1993). However, an advantage of using this *in vivo* system is that the experimentally-induced thrombus can be seeded with genetically modified cells, γ-Jagged oligomer, or soluble Jagged protein as described above for the *in vitro* approach, to influence two distinct phases of the angiogenic cascade in a controlled fashion.

The source of these endothelial cells is from large vessels, but they behave like capillaries when they migrate and form tubes, until some, but not all, will recruit pericytes and smooth muscle cells and assume the appearance and function of large vessels again. Clinically, both in the coronary and in the peripheral circulation, this revascularization process is critical, since successful recanalization of occluding thrombi is highly beneficial to the patient, but its regulation has been poorly understood.

Although an expert qualitative pathologic-anatomical evaluation of the vascular morphology is essential in these *in vivo* experiments, there are a number of time points that are amenable to quantitative morphometric analysis. This is especially relevant since these time points represent distinct stages in this process. At 4, 6, and 8 days, the number of invading cells are directly counted using a light microscope to evaluate cross-sections. Using immunohistochemical analysis with the CD34 antibody, the relative number of migrating endothelial cells is quantifiable; and using the leukocyte common antigen, the inflammatory cells can be assessed. Unfortunately, smooth muscle cell α-actin cannot be used as a reliable marker for myofibroblasts at this stage, since their phenotype is altered. However, by subtraction, the number of non-endothelial cells can be determined.

Thus, quantification of this early phase indicates whether, and in which direction, the interplay between Jagged and Notch influences the migratory component of the angiogenic process. Using serial sections of the same preparations, the proliferative cell nuclear antigen is useful to evaluate the relative contribution of proliferation to the total number of cells that populate the thrombus. When the thrombus is seeded with transfected cells expressing soluble Jagged, the <u>myc</u> reporter gene is used to recognize and count these components within the system.

Quantification of the functional vascular lumina in a cross-section after 2 and 4 weeks provides additional insight into the relationship between tube formation and the processes of endothelial migration and proliferation during angiogenesis. This comprises a statistical comparison of the number of individual lumina, grid point counts, and area measurements in perfused vessels. Mechanistically, the Jagged/Notch interaction which initiates tube formation from large vessel endothelial cells *in vitro*, may prove to be a stop signal for migration and proliferation of the microvasculature.

The endothelial cell site-specific effect of the Jagged-Notch system may also be responsible for the control and coordination of the migration/proliferation/tube formation sequence that ultimately leads to the formation of a new vessel. This can be shown *in vivo* in a revascularized thrombus murine model system, in which it is possible to deliberately exaggerate or compete with each of the components at the molecular level and at any time point within the process. Indeed, the kinetics of the Jagged/Notch interaction may also be assessable by seeding the thrombus at a later time point with soluble Jagged transfectants.

In the mouse, experimental intervention will involve a surgical exposure of previously treated, occluded carotid artery for an injection of a small volume of either lethally irradiated transfectants, recombinant protein or γ-Jagged oligomer into the site. However, the occluded vessel cannot bleed due to incomplete revascularization. Appropriate controls for the repetitive minor surgical trauma are possible in the same mammal on the contralateral carotid, using cells transfected with an inactive, but minimally altered mutant, inactive recombinant protein, or sense or inactive mutant γ-Jagged oligomers respectively.

While the model is useful to examine the formation of a new three-dimensional network of functioning vascular tubes, an additional model for the reendothelialization of the intima of a large vessel is needed, since Jagged/Notch appears to regulate this process in the opposite direction. Since murine vessels are too small for precise, selective de-endothelialization, the gently ballooned rat thoracic aorta (access from the carotid with a French 2 Edwards balloon) is an appropriate test system since it offers unequivocal starting points, and reasonably accurate quantification (*see*, Schwartz *et al.*, *Lab. Invest.* *38*:568-580 (1978)).

To assess the ability of the Jagged ligand to modify the migration of endothelial cells, thus influencing their ability to form a capillary network and/or to cover a de-endothelialized surface, one of several methods is indicated. In a first method, a therapeutically-effective

amount of soluble Jagged ligand is administered intravenously to mice and/or rats prior to and/or following thrombosis or balloon injury. In an alternative method, a thrombotic occlusion in a mouse is seeded with an effective amount of lethally irradiated NIH 3T3 cell soluble Jagged:myc transfectants. While in a third method, in both rats and mice, a distal site is seeded with an effective amount of lethally irradiated NIH 3T3 cell soluble Jagged:myc transfectants onto a subcutaneous fibrin matrix implant, which has been pretreated with lethally irradiated NIH 3T3 cells transfected with a hst-sp-FGF-1 construct using the nude mouse (Forough *et al.*, *J. Biol. Chem. 268*:2960-2968 (1993)).

It is known that the NIH 3T3 cells hst-sp-FGF-1 transfectants (10^5 cells) are able to secrete FGF-1 as an extracellular angiogenesis signal, and establish within 5 to 10 days an aggressive capillary network (Forough *et al.*, 1993). This is a result of the ligation of the signal peptide (sp) sequence from the hst/KS5 (FGF-4) gene to FGF-1, which directs the traffic of the hst-sp-FGF-1 chimera into the ER-Golgi apparatus for proteolytic processing of the hst/KS5-sp-sequence and release of FGF-1 as a soluble, extracellular protein. The efficacy of this construct has been established *in vivo* (Nabel et al., 1993; Robinson *et al.*, *Development 121*:505-514 (1995)).

In the third method, following thrombotic occlusion, the NIH 3T3 cell soluble Jagged:myc transfectants (10^6-10^7 cells) are injected into the angiogenic site, enabling the cells to express and secrete the soluble Jagged protein into the vasculature. The levels of plasma-derived Jagged (tail vein samples) are monitored by ELISA using the myc-epitope and Jagged antibodies. The rats are then assessed over time (*e.g.*, 1 to 10 days at 2 day intervals) for re--endothelialization of the denuded artery using Evan's blue staining. The degree of angiogenesis in the occlusion zone in the murine vessels is assessed using morphometric analysis of individual endothelial cells and of the fully developed capillary vessels in histological sections. Indeed, analysis by transmission electron microscopy will clearly demonstrate the involvement of endothelial cell migration and sprout formation in this system.

The assessment of the pharmacologic administration of intravenous soluble Jagged in the first method is based upon a similar end point, but utilizes a sufficient amount of recombinant Jagged to saturate both the Notch-1 and Notch-2 receptor Jagged-binding sites. The number and affinity of Jagged-binding sites on the surface of the murine endothelial cell are quantified *in vitro* by Scatchard analysis of murine aorta-derived endothelial cells and adipose-

derived microvascular endothelial cells using competitive (^{125}I)-Jagged binding by the method described for FGF-1 (Schreiber *et al.*, *Proc. Natl. Acad. Sci. USA 82*:6138-6142 (1985)).

The apparent lack of regulation of the Notch-1 and Notch-2 transcripts in the HUVEC population (Figure 5), predicts a high affinity Kd (pM) with approximately 5-20,000 Notch-binding sites per cell. The radiolabelling of the Jagged polypeptide utilizes the lactoperoxidase method, followed by removal of free (^{125}I) by Sephadex G-50 gel exclusion chromatography. This provides a pharmacologic range for the administration of the ligand. In addition, the availability of (^{125}I)-Jagged will demonstrate the expected pharmacokinetics of intravenous Jagged using methods previously successful for FGF-1 (Rosengart *et al.*, *Circ. Res. 64*:227-234 (1989).

In sum, these models should provide an *in vivo* correlate and *in vivo* models for Jagged function, demonstrating a predicted increase (25%-35%) in lumen re-endothelialization, and a similar decrease in the formation of capillary structures. In comparisons between the *in vivo* revascularization and reendothelialization experiments in normotensive animals, and in their spontaneously hypertensive rat counterparts (SHR, commercially available from Charles River with guaranteed hypertension), it has been shown that hypertension has a direct, albeit subtle, effect on the aortic endothelium of these model animals (Haudenschild *et al.*, *Hypertension 3*:148-153 (1981)). The aortic re-endothelialization experiments can be repeated in these rats without modification and with hypertension as the only added variable, however, the thrombus revascularization experiments must also be performed in these rats, since there is no comparable murine hypertension model available. The thrombi have been shown to be readily reproducible in mice, rats and rabbits. Thus, species differences do not pose a technical problem in the *in vivo* model systems.

Example 8

Expression of soluble jagged in the NIH 3T3 cell line

To determine the effects of a secreted, extracellular form of Jagged, a modified form of the Jagged gene was synthesized, transfected into the NIH 3T3 cell line, and then to select for those cells producing the protein. To track and monitor the fate of this Jagged molecule, a *myc* tag (reviewed by Kolodziej and Young, *Methods in Enzymology 194*:508-519 (1991)) was also introduced at the 3' end of the gene. In order to do this, several modifications of the

jagged gene were necessary, these are; (1) a Kozak sequence (Kozak, *J. Cell Biol. 108*:229-241 (1989)) was engineered onto the 5' end of the gene to ensure efficient transcription (2) a myc epitope tag placed at the 3' end (3) cloning sites engineered on both the 5' end (EcoR1, BamH1, Sal1 sites) and the 3' end (Xho1 site).

The primer pair used for this construction were:

5' end: Sense

GACTATGCGAATTCGGATCCGTCGACGCCACCATGG

Anti-sense 5' end: CAAGTTCCCCCGTTGAGACA

3' end myc tag construction

3' end/antisense primer

GCATAGTCCTCGAGTTACAAGTCTTCTTCAGAAATAAGCTTTTGTTCTACGATGT ACTCCATTCG

3' end/sense primer

ATGGACAAACACCAGCAGAA

Cycling reactions were as previously described in this application.

The 5' reaction was digested with EcoR1 and BglI1 , the 3' reaction was digested with Xho1 and Acc 1 site . These were ligated via standard protocol into a similarly digested Jagged template. The final gene product was then digested with EcoR1 and Xho1 and ligated into the eukaryotic expression vector pMexNeo2. This was then transfected into the NIH 3T3 cell line and cells grown in selection media containing G418 (as previously described).

Calcium mediated DNA was transferred into NIH 3T3 cells followed by growth in selective media results in clone: MW38-1.1 which synthesized the anticipated protein and also released it into the surrounding medium (conditioned media).

These 38-1.1 cells showed a unique phenotype. They grossly formed cord-like structures *in vitro* correlating with the presence of pseudo-lumens by ultrastructure analysis. In addition, they were able to induce wild type NIH to partially assume this phenotype. As such, 38-1.1 would be an outstanding resource both for the production and isolation of the

soluble Jagged (sol-jag) protein, and also for its ability to modulate the differentiation pattern of adjacent cells.

Although the present invention has been described with reference to the presently preferred embodiments, the skilled artisan will appreciate that various modifications, substitutions, omissions and changes may be made without departing from the spirit of the invention. Accordingly it is intended that the scope of the present invention be limited only by the scope of the following claims, including equivalents thereof.

jagged consensus Map (1 > 5458) Site and Sequence Settings: Linear, Certain Sites Only, Standard Genetic Code

```
GGCACGAGCCTAAGCCTCCTGCTCGCCCTGCTCTGTGCCCTGCGAGCCAAGGTGTGTGGGGCCTCGGGTCAGTTCGAGTTGGAGATCCTGTCCAT 95

 signal peptide

 G  T  S  L  S  L  L  L  A  L  L  C  A  L  R  A  K  V  C  G  A  S  G  Q  F  E  L  E  I  L  S  M

GCAGAACGTGAACGGGGAGCTGCAGAACGGGAACTGCTGCGGCGGCGCCCGGAACCCGGGAGACCGCAAGTGCACCCGCGACGAGTGTGACACAT 190

 Q  N  V  N  G  E  L  Q  N  G  N  C  C  G  G  A  R  N  P  G  D  R  K  C  T  R  D  E  C  D  T

ACTTCAAAGTGTGCCTCAAGGAGTATCAGTCCCGCGTCACGGCCGGGGGCCCTGCAGCTTCGGCTCAGGGTCCACGCCTGTCATCGGGGGCAAC 285

 Y  F  K  V  C  L  K  E  Y  Q  S  R  V  T  A  G  G  P  C  S  F  G  S  G  S  T  P  V  I  G  G  N

ACCTTCAACCTCAAGGCCAGCCGCGGCAACGACCGCAACCGCATCGTGCTGCCTTTCAGTTTCGCCTGGCCGAGGTCCTATACGTTGCTTGTGGA 380

 T  F  N  L  K  A  S  R  G  N  D  R  N  R  I  V  L  P  F  S  F  A  W  P  R  S  Y  T  L  L  V  E

GGCGTGGGATTCCAGTAATGACACCGTTCAACCTGACAGTATTATTGAAAAGGCTTCTCACTCGGGCATGATCAACCCCAGCCGGCAGTGGCAGA 475

 A  W  D  S  S  N  D  T  V  Q  P  D  S  I  I  E  K  A  S  H  S  G  M  I  N  P  S  R  Q  W  Q

CGCTGAAGCAGAACACGGGCGTTGCCCACTTTGAGTATCAGATCCGCGTGACCTGTGATGACTACTACTATGGCTTTGGCTGCAATAAGTTCTGC 570

                                                     DSL domain

 T  L  K  Q  N  T  G  V  A  H  F  E  Y  Q  I  R  V  T  C  D  D  Y  Y  Y  G  F  G  C  N  K  F  C
```

CGCCCCAGAGATGACTTCTTTGGACACTATGCCTGTGACCAGAATGGCAACAAAACTTGCATGGAAGGCTGGATGGGCCCCGAATGTAACAGAGC 665

DSL domain

R P R D D F F G H Y A C D Q N G N K T C M E G W M G P E C N R A

TATTTGCCGACAAGGCTGCAGTCCTAAGCATGGGTCTTGCAAACTCCCAGGTGACTGCAGGTGCCAGTATGGCTGGCAAGGCCTGTACTGTGATA 760

EGF repeats

I C R Q G C S P K H G S C K L P G D C R C Q Y G W Q G L Y C D

AGTGCATCCCACACCCGGGATGCGTCCACGGCATCTGTAATGAGCCCTGGCAGTGCCTCTGTGAGACCAACTGGGGCGGCCAGCTCTGTGACAAA 855

EGF repeats

K C I P H P G C V H G I C N E P W Q C L C E T N W G G Q L C D K

GATCTCAATTACTGTGGGACTCATCAGCCGTGTCTCAACGGGGGAACTTGTAGCAACACAGGCCCTGACAAATATCAGTGTTCCTGCCCTGAGGG 950

EGF repeats

D L N Y C G T H Q P C L N G G T C S N T G P D K Y Q C S C P E G

GTATTCAGGACCCAACTGTGAAATTGCTGAGCACGCCTGCCTCTCTGATCCCTGTCACAACAGAGGCAGCTGTAAGGAGACCTCCCTGGGCTTTG 1045

EGF repeats

Y S G P N C E I A E H A C L S D P C H N R G S C K E T S L G F

SUBSTITUTE SHEET (RULE 26)

AGTGTGAGTGTTCCCCAGGCTGGACCGGCCCCACATGCTCTACAAACATTGATGACTGTTCTCCTAATAACTGTTCCCACGGGGGCACCTGCCAG 1140

EGF repeats

E C E C S P G W T G P T C S T N I D D C S P N N C S H G G T C Q

GACCTGGTTAACGGATTTAAGTGTGTGTGCCCCCCACAGTGGACTGGGAAAACGTGCCAGTTAGATGCAAATGAATGTGAGGCCAAACCTTGTGT 1235

EGF repeats

D L V N G F K C V C P P Q W T G K T C Q L D A N E C E A K P C V

AAACGCCAAATCCTGTAAGAATCTCATTGCCAGCTACTACTGCGACTGTCTTCCCGGCTGGATGGGTCAGAATTGTGACATAAATATTAATGACT 1330

EGF repeats

N A K S C K N L I A S Y Y C D C L P G W M G Q N C D I N I N D

GCCTTGGCCAGTGTCAGAATGACGCCTCCTGTCGGGATTTGGTTAATGGTTATCGCTGTATCTGTCCACCTGGCTATGCAGGCGATCACTGTGAG 1425

EGF repeats

C L G Q C Q N D A S C R D L V N G Y R C I C P P G Y A G D H C E

AGAGACATCGATGAATGTGCCAGCAACCCCTGTTTGAATGGGGGTCACTGTCAGAATGAAATCAACAGATTCCAGTGTCTGTGTCCCACTGGTTT 1520

EGF repeats

R D I D E C A S N P C L N G G H C Q N E I N R F Q C L C P T G F

CTCTGGAAACCTCTGTCAGCTGGACATCGATTATTGTGAGCCTAATCCCTGCCAGANCGGTGCCCAGTGCTACAACCGTGCCAGTGACTATTTCT 1615

EGF repeats

S G N L C Q L D I D Y C E P N P C Q ? G A C C Y N R A S D Y F

GCAAGTGCCCCGAGGACTATGAGGGCAAGAACTGCTCACACCTGAAAGACCACTGCCGCACGACCCCCTGTGAAGTGATTGACAGCTGCACAGTG 1710

EGF repeats

C K C P E D Y E G K N C S H L K D H C R T T P C E V I D S C T V

GCCATGGCTTCCAACGACACACCTGAAGGGGTGCGGTATATTTCCTCCAACGTCTGTGGTCCTCACGGGAAGTGCAAGAGTCAGTCGGGAGGCAA 1805

EGF repeats

A M A S N D T P E G V R Y I S S N V C G P H G K C K S Q S G G K

ATTCACCTGTGACTGTAACAAAGGCTTCACGGGAACATACTGCCATGAAAATATTAATGACTGTGAGAGCAACCCTTGTAGAAACGGTGGCACTT 1900

EGF repeats

F T C D C N K G F T G T Y C H E N I N D C E S N P C R N G G T

GCATCGATGGTGTCAACTCCTACAAGTGCATCTGTAGTGACGGCTGGGAGGGGGCCTACTGTGAAACCAATATTAATGACTGCAGCCAGAACCCC 1995

EGF repeats

C I D G V N S Y K C I C S D G W E G A Y C E T N I N D C S Q N P

TGCCACAATGGGGGCACGTGTCGCGACCTGGTCAATGACTTCTACTGTGACTGTAAAAATGGGTGGAAAGGAAAGACCTGCCACTCACGTGACAG 2090

EGF repeats

C H N G G T C R D L V N D F Y C D C K N G W K G K T C H S R D S

TCAGTGTGATGAGGCCACGTGCAACAACGGTGGCACCTGCTATGATGAGGGGGATGCTTTTAAGTGCATGTGTCCTGGCGGCTGGGAAGGAACAA 2185

EGF repeats

Q C D E A T C N N G G T C Y D E G D A F K C M C P G G W E G T

CCTGTAACATAGCCCGAAACAGTAGCTGCCTGCCCAACCCCTGCCATAATGGGGGCACATGTGTGGTCAACGGCGAGTCCTTTACGTGCGTCTGC 2280

EGF repeats

T C N I A R N S S C L P N P C H N G G T C V V N G E S F T C V C

AAGGAAGGCTGGGAGGGGCCCATCTGTGCTCAGAATACCAATGACTGCAGCCCTCATCCCTGTTACAACAGCGGCACCTGTGTGGATGGAGACAA 2375

EGF repeats

K E G W E G P I C A Q N T N D C S P H P C Y N S G T C V D G D N

CTGGTACCGGTGCGAATGTGCCCCGGGTTTTGCTGGGCCCGACTGCAGAATAAACATCAATGAATGCCAGTCTTCACCTTGTGCCTTTGGAGCGA 2470

EGF repeats

W Y R C E C A P G F A G P D C R I N I N E C Q S S P C A F G A

CCTGTGTGGATGAGATCAATGGCTACCGGTGTGTCTGCCCTCCAGGGCACAGTGGTGCCAAGTGCCAGGAAGTTTCAGGGAGACCTTGCATCACC 2565

EGF repeats — cysteine-r-

T C V D E I N G Y R C V C P P G H S G A K C Q E V S G R P C I T

ATGGGGAGTGTGATACCAGATGGGGCCAAATGGGATGATGACTGTAATACCTGCCAGTGCCTGAATGGACGGATCGCCTGCTCAAAGGTCTGGTG 2660

cysteine-rich region

M G S V I P D G A K W D D D C N T C Q C L N G R I A C S K V W C

TGGCCCTCGACCTTGCCTGCTCCACAAAGGGCACAGCGAGTGCCCCAGCGGGCAGAGCTGCATCCCCATCCTGGACGACCAGTGCTTCGTCCACC 2755

cysteine-rich region

G P R P C L L H K G H S E C P S G Q S C I P I L D D Q C F V H

CCTGCACTGGTGTGGGCGAGTGTCGGTCTTCCAGTCTCCAGCCGGTGAAGACAAAGTGCACCTCTGACTCCTATTACCAGGATAACTGTGCGAAC 2850

cysteine-rich region

P C T G V G E C R S S S L Q P V K T K C T S D S Y Y Q D N C A N

ATCACATTTACCTTTAACAAGGAGATGATGTCACCAGGTCTTACTACGGAGCACATTTGCAGTGAATTGAGGAATTTGAATATTTTGAAGAATGT 2945

cysteine-rich region

I T F T F N K E M M S P G L T T E H I C S E L R N L N I L K N V

TTCCGCTGAATATTCAATCTACATCGCTTGCGAGCCTTCCCCTTCAGCGAACAATGAAATACATGTGGCCATTTCTGCTGAAGATATACGGGATG 3040

cysteine-rich region

S A E Y S I Y I A C E P S P S A N N E I H V A I S A E D I R D

ATGGGAACCCGATCAAGGAAATCACTGACAAAATAATCGATCTTGTTAGTAAACGTGATGGAAACAGCTCGCTGATTGCTGCCGTTGCAGAAGTA 3135

D G N P I K E I T D K I I D L V S K R D G N S S L I A A V A E V

AGAGTTCAGAGGCGGCCTCTGAAGAACAGAACAGATTTCCTTGTTCCCTTGCTGAGCTCTGTCTTAACTGTGGCTTGGATCTGTTGCTTGGTGAC 3230

transmembrane domain

R V Q R R P L K N R T D F L V P L L S S V L T V A W I C C L V T

GGCCTTCTACTGGTGCCTGCGGAAGCGGCGGAAGCCGGGCAGCCACACACACTCAGCCTCTGAGGACAACACCACCAACAACGTGCGGGAGCAGC 3325

-transmembrane domain cytoplasmic region

A F Y W C L R K R R K P G S H T H S A S E D N T T N N V R E Q

TGAACCAGATCAAAAACCCCATTGAGAAACATGGGGCCAACACGGTCCCCATCAAGGATTACGAGAACAAGAACTCCAAAATGTCTAAAATAAGG 3420

cytoplasmic region

L N Q I K N P I E K H G A N T V P I K D Y E N K N S K M S K I R

ACACACAATTCTGAAGTAGAAGAGGACGACATGGACAAACACCAGCAGAAAGCCCGGTTTGGCAAGCAGCCGGCGTTTTCGCTGGTAGACAGAGA 3515

cytoplasmic region

T H N S E V E E D D M D K H Q Q K A R F G K Q P A F S L V D R E

AGAGAAGCCCCCCAACGGCACGCCGACAAAACACCCAAACTGGACAAACAAACAGGACAACAGAGACTTGGAAAGTGCCCAGAGCTTAAACCGAA 3610

cytoplasmic region

E K P P N G T P T K H P N W T N K Q D N R D L E S A Q S L N R

TGGAGTACATCGTATAGCAGACCGCGGGCACTGCCGCCGCTAGGTAGAGTCTGAGGGCTTGTAGTTCTTTAAACTGTCGTGTCATACTCGAGTCT 3705

·cytoplasmic region

M E Y I V

GAGGCCGTTGCTGACTTAGAATCCCTGTGTTAATTTAAGTTTTGACAAGCTGGCTTACACTGGCAATGGTAGTTTCTGKGGTTGGCTGGGAAATC 3800

GAGTGCCGCATCTCACAGCTATGCAAAAAGCTAGTCAACAGTACCCTGGTTGTGTGTCCCCTTGCAGCCGACACGGTCTCGGATCAGGCTCCCAG 3895

GAGCCTGCCCAGCCCCCTGGTCTTTGAGCTCCCACTTCTGCCAGATGTCCTAATGGTGATGCAGTCTTAGATCATAGTTTTATTTATATTTATTG 3990

ACTCTTGAGTTGTTTTTGTATATTGGTTTTATGATGACGNACAAGTAGTTCTGTATTTGAAAGTGCCTTTGCAGCTCAGAACCACAGCAACGATC 4085

ACAAATGACTTTATTATTTATTTTTTTAATTGTATTTTTGTTGTTGGGGGAGGGGAGACTTTGATGTCAGCAGTTGCTGGTAAAATGAAGAATTT 4180

AAAGAARAAAATGTCMMMNAGTAGAACTTTGTATAGTTATGTAAATAATTCTTTTTTATTAATCACTGTGTATATTTGATTAKATTAAMTTAATA 4275

ATCAAGAGCCTTAAAACATCATTCCTTTTTATTTATATGTATGTGTTTAGAATTGNAGGTTTTTGATAGCATTGTAAGCGTATGGCTTTATTTTT 4370

TTGAACTCTTCTCATTACTTGTTGCCTATAAGCCMMMATTAAGGTGTTTGAAAATAGTTTATTTTAAAACAATAGGATGGGCTTCTGTGCCCAGA 4465

ATACTGATGGAATTTTTTTTGTACGACGTCAGATGTTTAAAACACCTTCTATAGCATCACTTAAAACACGTTTTAAGGACTGACTGAGGCAGTTT 4560

GAGGATTAGTTTAGAACAGGTTTTTTTGTTTGTTTGTTTTTTGTTTTTCTGCTTTAGACTTGAAAAGAGACAGGCAGGTGATCTGCTGCAGAGCA 4655

GTAAGGGAACAAGTTGAGCTATGACTTAACATAGCCAAAATGTGAGTGGTTGAATATGATTAAAAATATCAAATTAATTGTGTGAACTTGGAAGC 4750

ACACCAATCTGACTTTGTAAATTCTGATTTCTTTTCACCATTCGTACATAATACTGAACCACTTGTAGATTTGATTTTTTTTTTAATCTACTGCA 4845

TTTAGGGAGTATTCTAATAAGCTAGTTGAATACTTGAACCATAAAATGTCCAGTAAGATCACTGTTTAGATTTGCCATAGAGTACACTGCCTGCC 4940

TTAAGTGAGGAAATCAAAGTGCTATTACGAAGTTCAAGATCMAAAAGGCTTATAAAACAGAGTAATCTTGTTGGTTCACCATTGAGACCGTGAAG 5035

ATACTTTGTATTGTCCTATTAGTGTTATATGAACABACAAATGCATCTTTGATGTGTTGTTCTTGGCAATAAATTTTGAAAAGTAATATTTATTA 5130

AATTTTTTTGTATGAAAACATGGAACAGTGTGGCCTCTTCTGAGCTTACGTAGTTCTACCGGCTTTGCCGTGTGCTTCTGCCACCCTGCTGAGTC 5225

TGTTCTGGTAATCGGGGTATAATAGGCTCTGCCTGACAGAGGGATGGAGGAAGAACTGAAAGGCTTTTCAACCACAAAACTCATCTGGAGTTCTC 5320

AAAGACCTGGGGCTGCTGTGAAGCTGGAACTGCGGGAGCCCCATCTAGGGGAGCCTTGATTCCCTTGTTATTCAACAGCAAGTGTGAATACTGCT 5415

TGAATAAACACCACTGGATTAAAAAAAAAAAAAAAAAAAGGCA 4

SUBSTITUTE SHEET (RULE 26)

What is claimed is:

1. A substantially purified Jagged protein.

2. The protein according to claim 1, which is free of the proteins with which it is normally associated, and which has an amino acid sequence corresponding to SEQ ID NO:1.

3. The protein according to claim 2, wherein the protein comprises the amino acid sequence corresponding to SEQ ID NO:1, or functionally equivalent derivative, or allelic or species variant thereof.

4. The protein according to claim 3, wherein the protein comprises the amino acid sequence corresponding to SEQ ID NO:1, or functionally equivalent derivative, or allelic or species variant.thereof, and which is characterized by the ability to bind to Notch.

5. A substantially purified nucleic acid molecule or segment thereof encoding a Jagged protein, or functionally equivalent derivative, or allelic or species variant thereof.

6. The nucleic acid molecule according to claim 5, wherein the nucleic acid comprises the sequence corresponding to SEQ ID NO:1, or a segment thereof.

7. The nucleic acid molecule according to claim 6, wherein the nucleic acid comprises the nucleic acid sequence or segment thereof corresponding to SEQ ID NO:1, or functionally equivalent derivative, or allelic or species variant thereof.

8. The nucleic acid molecule according to claim 6, wherein the nucleic acid comprises the nucleic acid sequence or segment thereof corresponding to SEQ ID NO:1, or functionally equivalent derivative, or allelic or species variant thereof, and which is characterized by the ability to bind to Notch.

9. A recombinant molecule comprising a vector and the nucleic acid sequence according to claim 5.

10. A host cell comprising the recombinant molecule according to claim 9.

11. The expression product of the recombinant molecule according to claim 9.

12. A substantially purified, single-stranded, nucleic acid molecule comprising the antisense strand of the Jagged cDNA, or a segment thereof.

13. The nucleic acid molecule according to claim 12, wherein the nucleic acid comprises the antisense nucleotide sequence corresponding to SEQ ID NO:1, or a segment thereof.

14. The nucleic acid molecule according to claim 13, wherein the antisense nucleic acid comprises the antisense nucleic acid sequence corresponding to SEQ ID NO:1, or a segment thereof which if read in the sense direction would encode a functionally equivalent derivative, or allelic or species variant thereof.

15. The nucleic acid molecule according to claim 14, wherein the antisense nucleic acid comprises the antisense nucleic acid sequence corresponding to SEQ ID NO:1, or a segment thereof which if read in the sense direction would encode a functionally equivalent derivative, or allelic or species variant thereof, and which is characterized by the ability to bind to Jagged.

16. The polypeptide encoded by the nucleic acid molecule according to claims 12-15.

17. The polypeptide encoded by the nucleic acid molecule according to claims 12-15, wherein the polypeptide has a binding affinity to, and inhibits the activity of Jagged.

18. An antibody having a binding affinity to Jagged, or a unique portion thereof.

19. A secondary antibody having a binding affinity to anti-Jagged, or a unique portion thereof.

20. A method of decreasing the migration of endothelial cells to a site on a micro-diameter blood vessel, comprising delivering the protein of claims 1 or 19 to said site from which the endothelial cells have been removed, damaged or substantially reduced.

21. A method of increasing the migration of endothelial cells to a site on a macro-diameter blood vessel, comprising delivering the protein of claims 1 or 19 to said site from which the endothelial cells have been removed, damaged or substantially reduced.

22. A method of increasing the migration of endothelial cells to a site on a micro-diameter blood vessel, comprising delivering the protein of claims 16, 17 or 18 to said site from which the endothelial cells have been removed, damaged or substantially reduced.

23. A method of decreasing the migration of endothelial cells to a site on a macro-diameter blood vessel, comprising delivering the protein of claims 16, 17 or 18 to said site from which the endothelial cells have been removed, damaged or substantially reduced.

24. A method of decreasing the migration of smooth muscle cells to a site on a macro-diameter blood vessel, comprising delivering the protein of claims 16, 17 or 18 to said site from which the endothelial cells have been removed, damaged or substantially reduced.

25. A pharmaceutical composition comprising a therapeutically effective amount of a Jagged protein, or functionally equivalent derivative, or allelic or species variant thereof; and a pharmaceutically acceptable carrier.

26. A pharmaceutical composition comprising a therapeutically effective amount of a Jagged nucleic acid, or functionally equivalent derivative, or allelic or species variant thereof; and a pharmaceutically acceptable carrier.

27. A pharmaceutical composition comprising a therapeutically effective amount of a Jagged antibody, or functionally equivalent derivative, or allelic or species variant thereof; and a pharmaceutically acceptable carrier.

28. A pharmaceutical composition comprising a therapeutically effective amount of a Jagged antisense molecule, or functionally equivalent derivative, or allelic or species variant thereof; and a pharmaceutically acceptable carrier.

29. A pharmaceutical composition comprising a therapeutically effective amount of an anti-Jagged antibody, or functionally equivalent derivative, or allelic or species variant thereof; and a pharmaceutically acceptable carrier.

30. A method of preventing or treating a disease or condition in a subject comprising administering to a subject in need of such prevention or treatment a therapeutically effective amount of a molecule which antagonizes, inhibits or prevents the function of the Notch protein.

31. A method of preventing or treating a disease or condition in a subject comprising administering to a subject in need of such prevention or treatment a therapeutically effective amount of a molecule which agonizes, enhances or stimulates the function of the Notch protein.

32. A method of preventing or treating a disease or condition in a subject comprising administering to a subject in need of such prevention or treatment a therapeutically effective amount of a molecule which antagonizes, inhibits or prevents the function of the Jagged protein.

33. A method of preventing or treating a disease or condition in a subject comprising administering to a subject in need of such prevention or treatment a therapeutically effective amount of a molecule which agonizes, enhances or stimulates the function of the Notch protein.

34. A method of inhibiting or preventing angiogenesis in a subject comprising administering to a subject in need of such inhibition or prevention a therapeutically effective amount of a Jagged or a Jagged agonist.

35. The method according to claim 34, wherein the angiogenesis being inhibited or prevented comprises solid tumor angiogenesis.

36. The method according to claim 34, wherein the angiogenesis being inhibited or prevented comprises rheumatoid arthritic angiogenesis.

37. The method according to claim 34, wherein the angiogenesis being inhibited or prevented comprises inflammatory angiogenesis.

38. The method according to claim 34, wherein the angiogenesis being inhibited or prevented comprises restenosis of the lumen of a blood vessel.

39. The method according to claim 38, wherein the restenosis is inhibited or prevented by repressing angiogenesis from the vaso vasorum, and by promoting large vessel endothelial cell migration to repair the lumen of a large blood vessel.

40. The method according to claim 34, wherein the Jagged agonists comprise agents which promote the expression of Jagged, including fibrin and functional derivatives thereof and pharmacologically acceptable chemicals, and γ-idiotypic Jagged antibodies.

41. The method according to claim 34, wherein the inhibition or prevention occurs *in vivo* or *in vitro*.

42. A method of promoting or enhancing angiogenesis in a subject comprising administering to a subject in need of such promotion or enhancement a therapeutically effective amount of anti-Jagged or a Jagged antagonist.

43. The method according to claim 42, wherein the angiogenesis being promoted or enhanced comprises wound or injury repair angiogenesis.

44. The method according to claim 43, wherein the wound or injury being repaired was caused by surgery, trauma and/or disease or condition.

45. The method according to claim 44, wherein the disease or condition is diabetes-related.

46. The method according to claim 42, wherein the Jagged antagonists comprise Jagged antibodies, anti-sense Jagged, Jagged mutants and pharmacologically acceptable chemicals.

47. The method according to claim 42, wherein the promotion or enhancement occurs *in vivo* or *in vitro*.

48. A method of affecting cell differentiation of cells comprising the mesoderm, endoderm, ectoderm and/or neuroderm.

49. The method according to claim 48, wherein the cell types affected comprise hematopoietic stem cells, epithelial cells, vascular smooth muscle cells and dendritic cells.

50. A pharmaceutical composition used in any method of claims 30-48.

Figure 1

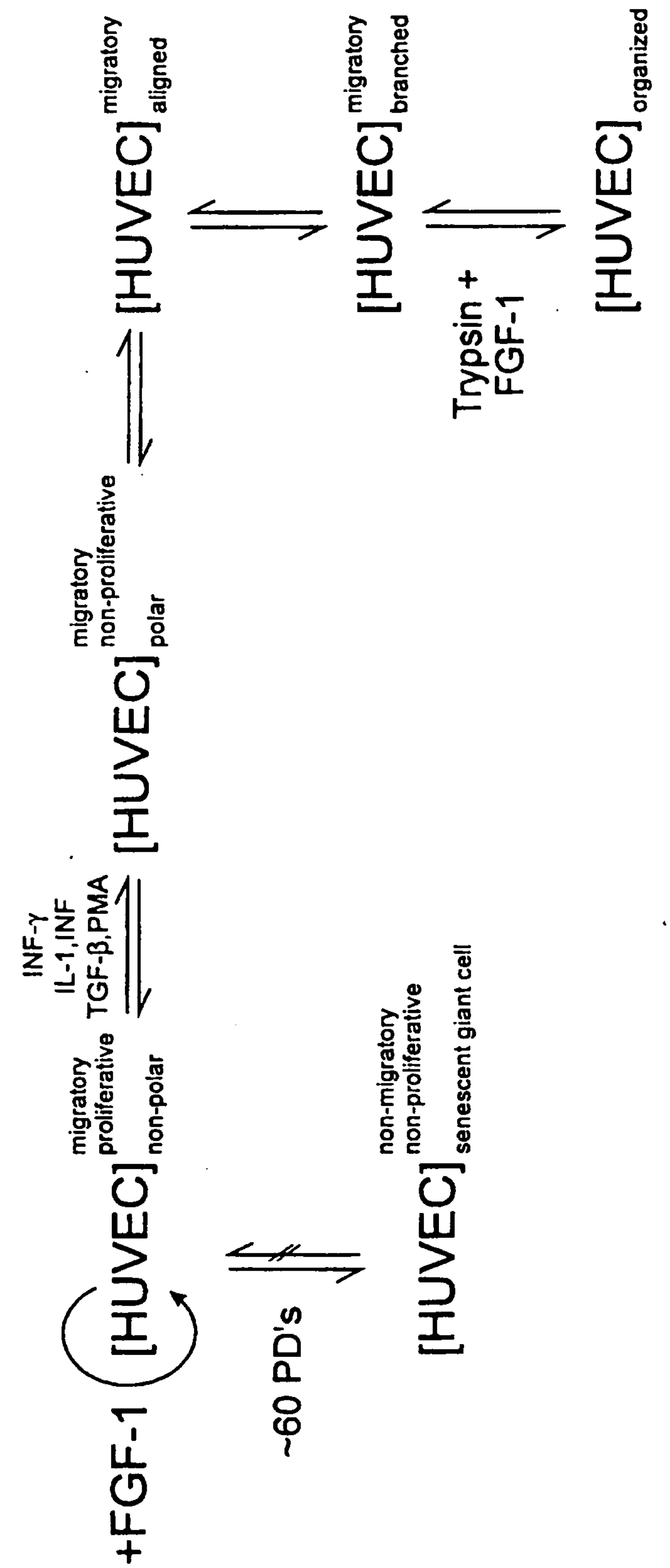

+FGF-1
[HUVEC] migratory proliferative non-polar
INF-γ
IL-1,INF
TGF-β,PMA
[HUVEC] migratory non-proliferative polar
[HUVEC] migratory aligned
[HUVEC] migratory branched
Trypsin +
FGF-1
[HUVEC] organized
~60 PD's
[HUVEC] non-migratory non-proliferative senescent giant cell

Figure 2

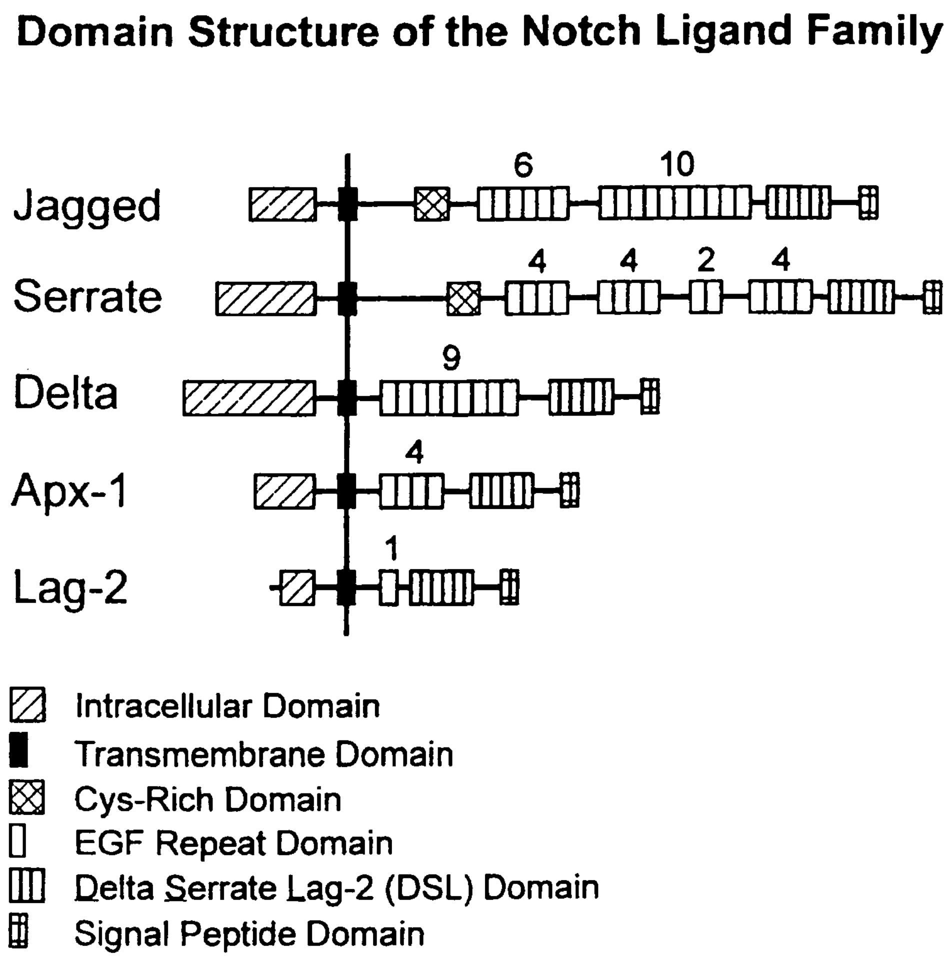

Domain Structure of the Notch Ligand Family
Jagged
Serrate
Delta
Apx-1
Lag-2
6
10
4
4
2
4
9
4
1
Intracellular Domain
Transmembrane Domain
Cys-Rich Domain
EGF Repeat Domain
Delta Serrate Lag-2 (DSL) Domain
Signal Peptide Domain

Figure 3

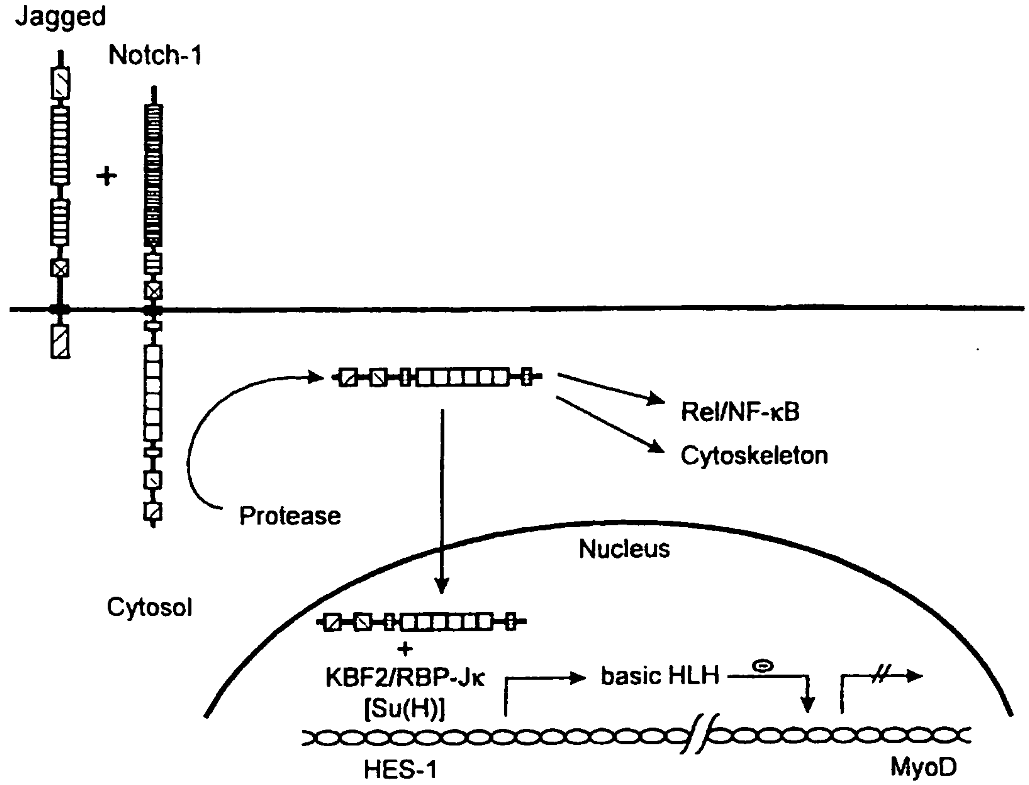
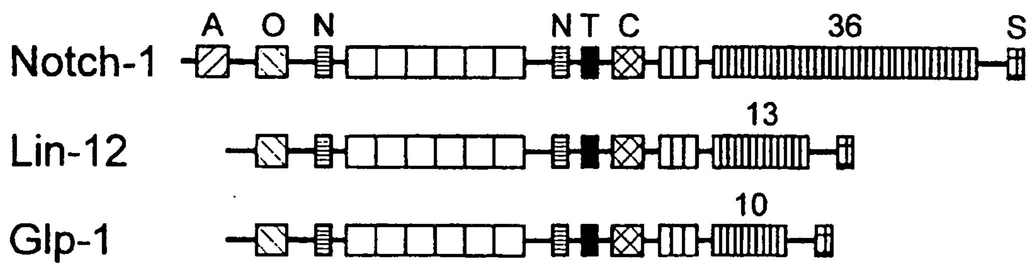

Jagged
Notch-1
+
Rel/NF-κB
Cytoskeleton
Protease
Nucleus
Cytosol
+
KBF2/RBP-Jκ
[Su(H)]
basic HLH
HES-1
MyoD

Figure 4

Domain Structure of the Notch Receptor Family



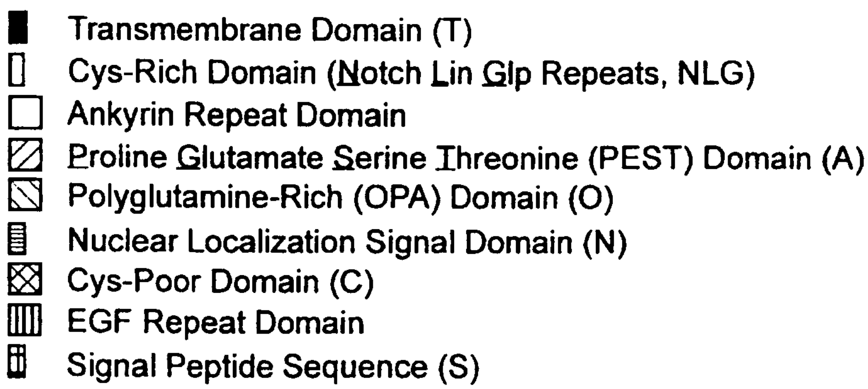


Figure 5

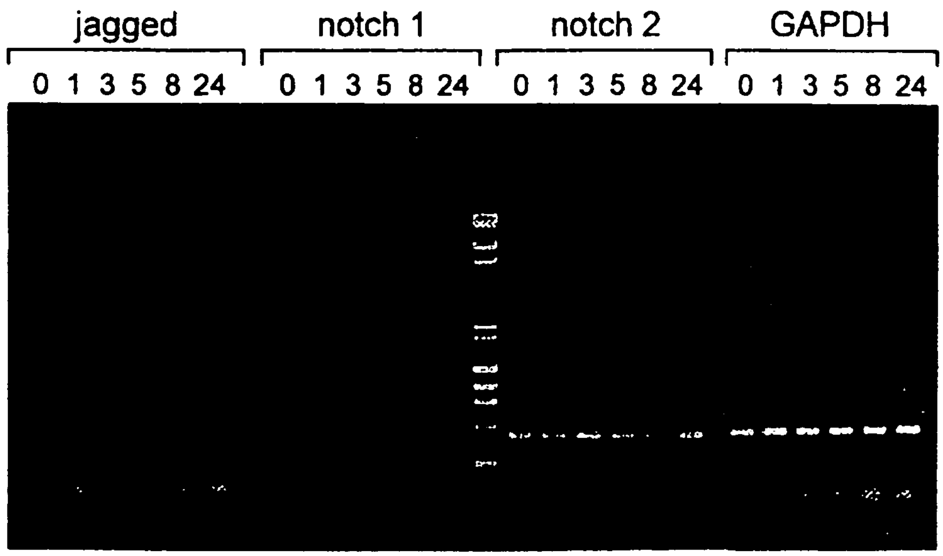

jagged
0 1 3 5 8 24
notch 1
0 1 3 5 8 24
notch 2
0 1 3 5 8 24
GAPDH
0 1 3 5 8 24

Figure 6

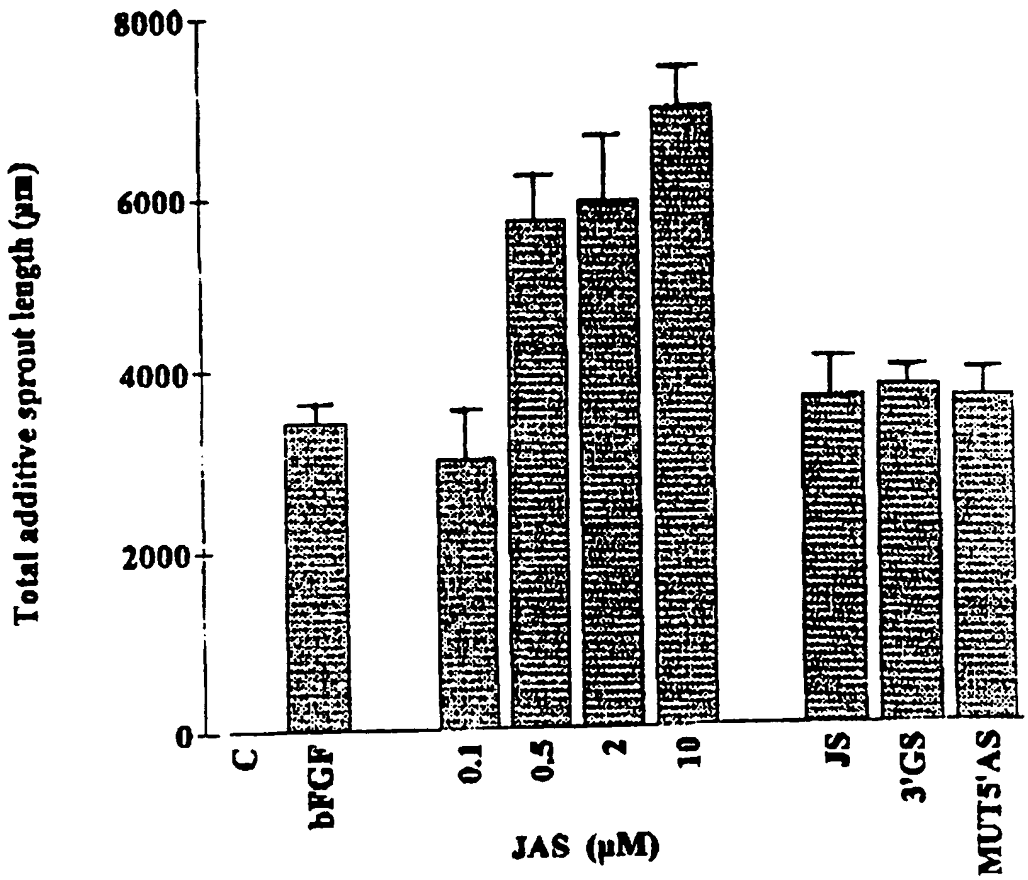

8000
6000
4000
2000
0
Total additive sprout length (µm)
C
bFGF
0.1
0.5
2
10
JS
3'GS
MUT5'AS
JAS (µM)

Figure 7A

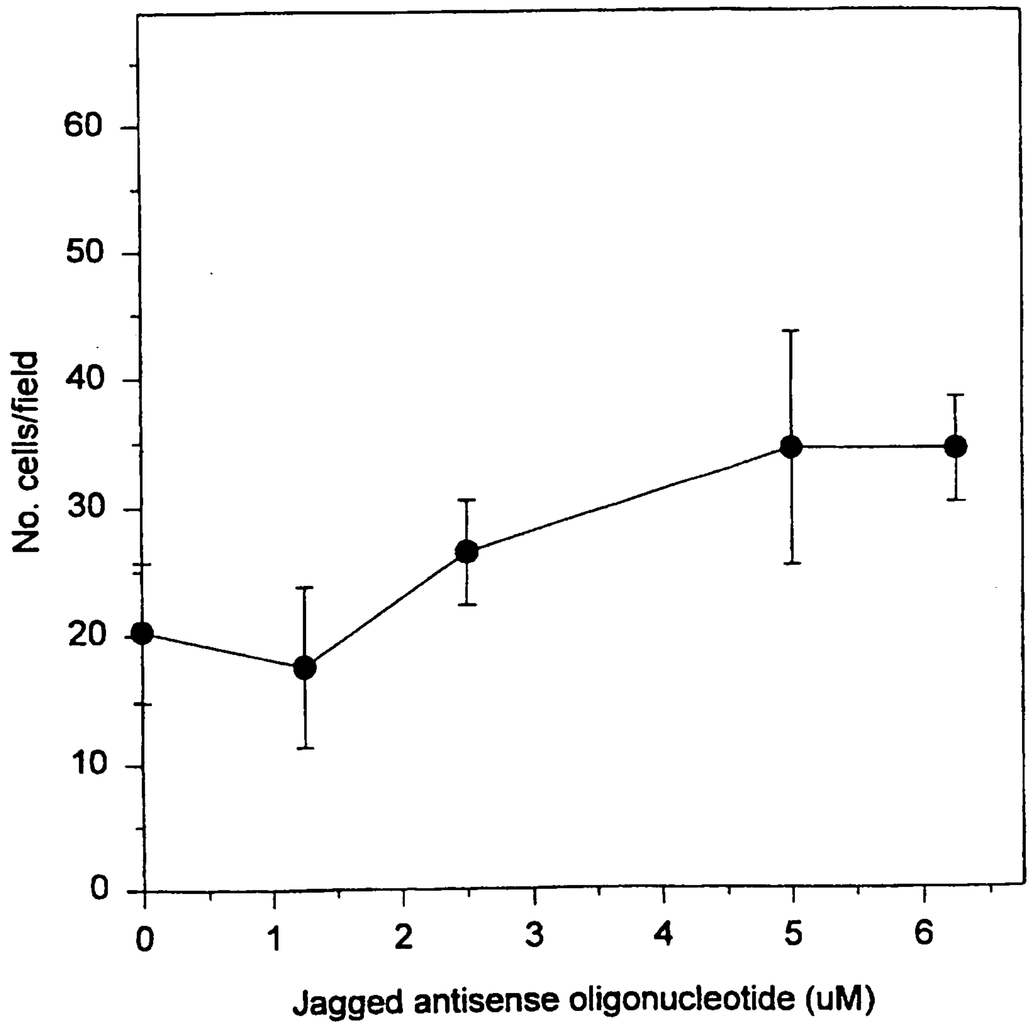
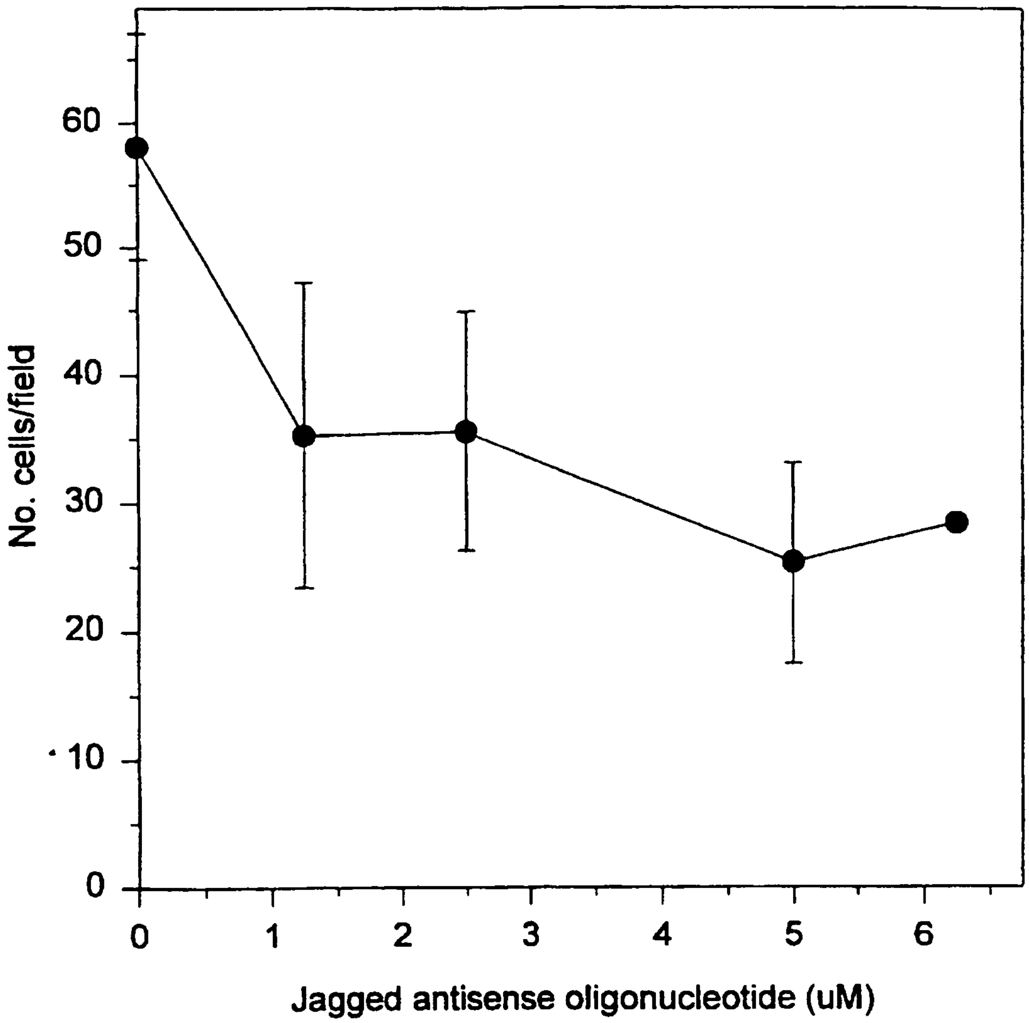

No. cells/field
0
10
20
30
40
50
60
0
1
2
3
4
5
6
Jagged antisense oligonucleotide (uM)

Figure 7B


60
50
40
30
20
10
0
No. cells/field
0
1
2
3
4
5
6
Jagged antisense oligonucleotide (uM)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09407

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, medline, embase, biosis, scisearch
jagged, notch ligand, angio?, endothel###

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	LINDSELL, C.E. et al. Jagged: A mammalian ligand that activates Notch1. Cell. 24 March 1995, Vol. 80, pages 909-917, see entire document.	1, 5, 9-11, 25-26 -------- 18-19, 27, 29 -------- 20-24, 30-50
X, P --- Y, P	ZIMRIN, A.B. et al. An antisense oligonucleotide to the notch ligand jagged enhances fibroblast growth factor-induced angiogenesis *in vitro*. J. Biol. Chem. 20 December 1996, Vol. 271, No. 51, pages 32499-32502, see entire document.	12, 16-17, 28 -------- 30-33, 42, 47-50

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 JULY 1997	20 AUG 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	F. PIERRE VANDERVEGT
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZIMRIN, A.B. et al. An antisense olignucleotide to the notch ligand jagged promotes angiogenesis in bovine microvascular endothelial cells (BMEC) on collagen gels. FASEB J. 30 April 1996, Vol. 10, No. 6, page A1094, abstract no. 547, see entire abstract.	1-50
Y	JOHNSTONE, A. et al. Immunochemistry in practice. Oxford, UK: Blackwell Scientific Publications, 1987, second edition, chapter 2 'Production of Antibodies', pages 30-47, see entire chapter.	18-24, 27, 29, 32, 42-50
A	HARRIS, W.J. et al. Therapeutic antibodies -- the coming of age. TIBTECH. February 1993, Vol. 11, pages 42-44, see entire document.	32, 42-50

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09407

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 48/00, 38/17, 38/18; C07K 14/435, 14/485, 16/28; C12N 15/11; C07H 21/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/350, 387.2, 388.2; 536/23.5, 24.5; 435/ 320.1, 69.1, 70.1, 325; 514/2, 44

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

530/350, 387.2, 388.2; 536/23.5, 24.5; 435/ 320.1, 69.1, 70.1, 325; 514/2, 44